MITOCHONDRIA: HUBS OF CELLULAR SIGNALING, ENERGETICS AND REDOX BALANCE

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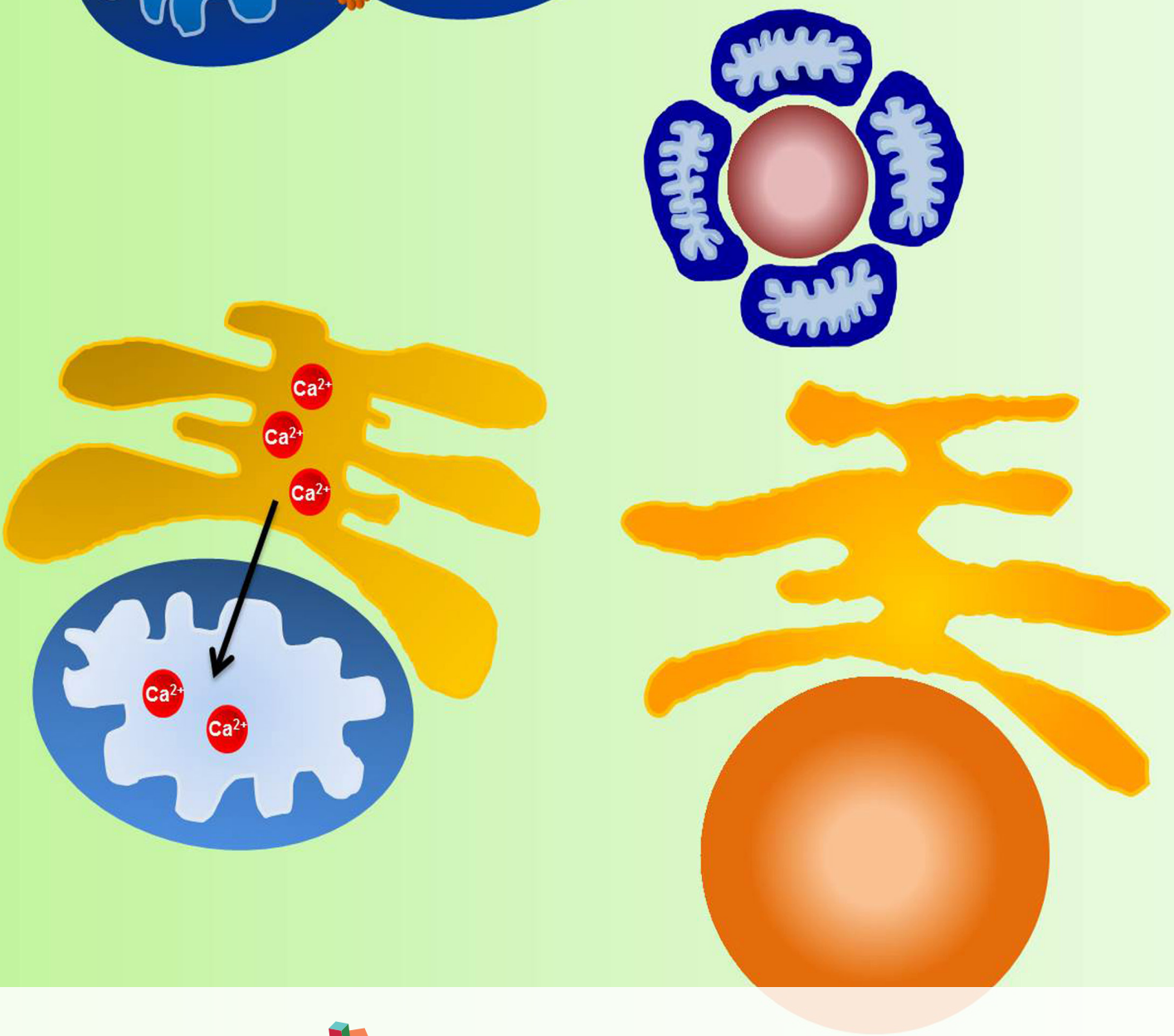

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MITOCHONDRIA: HUBS OF CELLULAR SIGNALING, ENERGETICS AND REDOX BALANCE

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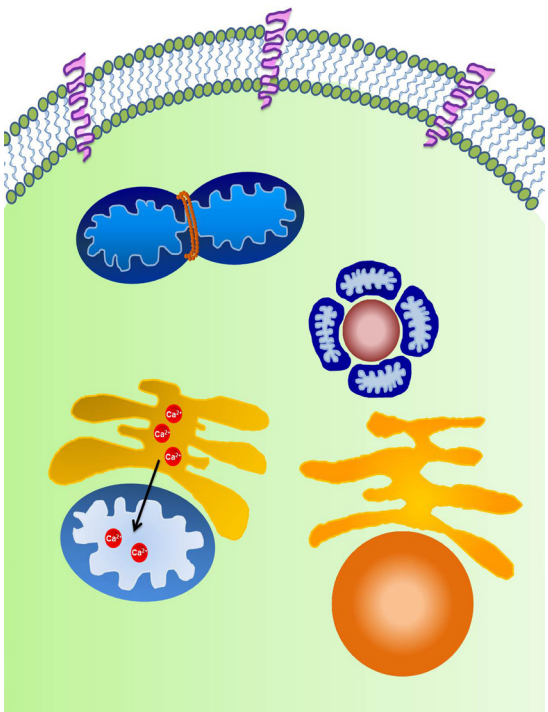

Mitochondria are dynamic organelles structurally and functionally connected with other cellular compartments. The mitochondrial network continuously undergoes fusion and fission. Close proximity and physical interaction between mitochondria and ER influences homeostasis of both organelles. Other interactions include association of mitochondria with lipid droplets or modulation of nuclear transcriptional response through retrograde signaling.

Figure by Soni Deshwal and Nina Kaludercic. Figure was taken and modified from Kaludercic N, Deshwal S and Di Lisa F (2014) Reactive oxygen species and redox compartmentalization. Front. Physiol. 5:285. doi:10.3389/fphys.2014.00285

Poised at the convergence of most catabolic and anabolic pathways, mitochondria are the center of heterotrophic aerobic life, representing a hub in the overall metabolic network of cells. The energetic functions performed by mitochondria face the unavoidable redox hurdle of handling huge amounts of oxygen while keeping its own as well as the cellular redox environment under control.

Reactive oxygen species (ROS) are produced in the respiratory chain as a result of the energy supplying function of mitochondria. Originally considered an unavoidable by-product of oxidative phosphorylation, ROS have become crucial signaling molecules when their levels are kept within physiological range. This occurs when their production and scavenging are balanced within mitochondria and cells.

Mitochondria-generated hydrogen peroxide can act as a signaling molecule within mitochondria or in the cytoplasm, affecting multiple networks that control, for example, cell cycle, stress response, cell migration and adhesion, energy metabolism, redox balance, cell contraction, and ion channels. However, under pathophysiological conditions, excessive ROS levels can happen due to either overproduction, overwhelming of antioxidant defenses, or both. Under oxidative stress, detrimental effects of ROS include oxidation of protein, lipids, and nucleic acids; mitochondrial depolarization and calcium overload; and cell-wide oscillations mediated by ROS-induced ROS release mechanisms.

Mitochondrial dysfunction is central in the pathogenesis of numerous human maladies including cardiomyopathies and neurodegeneration. Diseases characterized by altered nutrient metabolism, such as diabetes and cancer, exhibit elevated ROS levels. These may contribute to pathogenesis by increasing DNA mutation, affecting regulatory signaling and transcription, and promoting inflammation. Under metabolic stress, several ionic channels present in the inner and outer mitochondrial membranes can have pro-life and -death effects.

In the present E-book, based on the Frontiers Research Topic entitled: "Mitochondria: Hubs of cellular signaling, energetics and redox balance", we address one of the fundamental questions that the field of ROS biology faces today: how do mitochondria accomplish a reliable energy provision and at the same time keep ROS levels within physiological, non-harming, limits but crucial for cellular signaling function? Additionally, and within the perspective of mitochondria as signaling-energetic hubs in the extensive cellular metabolic network, we ask how can their collective dynamics scale from the subcellular to the cellular, tissue and organ levels to affect function in health and disease.

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EDITORIAL
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Mitochondria: hubs of cellular signaling, energetics and redox balance. A rich, vibrant, and diverse landscape of mitochondrial research

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Mitochondria have become the cornerstone of cellular biology, opening new frontiers in health and disease. Poised at the convergence of most catabolic and anabolic pathways, mitochondria constitute the center of heterotrophic aerobic life, processing and generating key metabolites that feed into pathways leading to growth, division, and signaling. As such, mitochondria play the role of hubs in the overall metabolic network; thus their failure risks the collapse of most crucial cellular functions. The protagonist role of mitochondria is underscored by the existence of tightly regulated cellular processes that include autophagy/mitophagy and metabolically tuned morphological changes induced by fusion-fission dynamics. Mitochondria are also involved in a myriad of signaling cascades regulating cell survival vs. death. They provide the energy for the cell, a hub for biosynthetic processes and they contain a self-destructive arsenal of apoptotogenic factors that can be unleashed to promote apoptotic signaling. Consequently, it is no wonder that mitochondrial dysfunction is implicated in the aging phenomenon, and in numerous human maladies including metabolic disorders, cardiomyopathies, and neurodegeneration. Indeed, diseases characterized by altered gene-nutrient interactions, such as diabetes and cancer, exhibit elevated levels of reactive oxygen species (ROS) of which mitochondria are a major source. Therefore, mitochondrial dysfunction and the resulting oxidative stress are central in these and other human pathologies. Excess ROS induced by oxidative stress are specifically known to contribute to these pathogeneses in part by increasing mitochondrial DNA mutations that, via retrograde signaling, affects nuclear gene expression, ultimately modulating gene transcription, protein translation, post-translational modifications, and cellular signaling.

For this Research Topic, our initial focus was on the younger generation of researchers working on mitochondria, believing both in their responsibility to take the next steps in this field, confident in their ability to take mitochondrial research to new and exhilarating heights. In this endeavor, 18 papers, including a Commentary to one of the contributions, reveal an exciting, broad scope of subjects involving mitochondrial research that exploit a variety of novel methods, insights, and ideas. The potential implications of mitochondria at the crossroad of cellular injury and therapeutics are also addressed.

Fresh insights into actively investigated areas affecting mitochondrial/cellular redox balance are highlighted in a series of review articles, as applied to the etiology of heart disease and insulin resistance (Alleman et al., 2014), and the role of the renin angiotensin system (Vajapey et al., 2014). The role of cellular redox compartmentalization (matrix vs. extra-matrix) in leading mitochondrial function from normal to pathological conditions (Kembro et al., 2014),

and the functional significance of the differential redox status exhibited by subcellular organelles (likely lysosomes, peroxisomes, endoplasmic reticulum, and nuclei) apart from mitochondria (Kaludercic et al., 2014) are also reported.

A review contribution by Birkedal et al. (2014), and an original research article by Kurz et al. (2014) highlight the use of new conceptual and computational tools to address intracellular energetic compartmentalization and mitochondrial network organization as essential components of the coordinated energetic response during the cardiac systole-diastole cycle. Hypertrophic cardiomyopathy, the most common inherited cardiac disease, is analyzed in a review by Vakrou and Abraham (2014) and defined as a metabolic disease in which mitochondrial function plays a relevant role.

A novel experiment on the protective role of infrared light against cardiac injury elicited under ischemia and reperfusion (IR) is presented in Keszler et al. (2014) and highlighted in a Commentary article by Karam and Akar (2014). In another study, Chen et al. (2014) report on the flavin-NADH-containing apoptosis-inducing factor (AIF), found in mitochondria and required for optimal respiratory function. AIF acts as a stimulator of ROS-mediated cell death during IR in the Harlequin mouse model that expresses AIF in reduced amounts. Additional studies using intact heart, analyze the role of mitochondrial ion channels in arrhythmic propensity under oxidative stress, during IR injury (Xie et al., 2014). Whereas low levels of ROS can serve as critical signaling molecules, excess ROS are implicated in IR injury. Lindsay et al. (2015) report on specific electron transport chain (ETC) complexes that are responsible for ROS generation under conditions that may prevail during prolonged ischemia. These include different substrate utilization, excessive mitochondrial calcium load and change in pH that culminate in mitochondrial permeability transition pore opening and ROS emission.

Signaling aspects related to mitochondrial activity also had a prominent contribution in this Research Topic. Protective strategies of mitochondrial energetic function in neurons based on Bcl-xL, a member of the anti-apoptotic Bcl-2 protein family, and directed to prevent cell death via mitochondrial permeability transition pore opening and outer membrane permeabilization, are addressed by Jonas et al. (2014). In a review article Papanicolaou et al. (2014) discussed the new emerging family of mitochondrial proteins that are post-translationally modified via direct reaction of lysine residues with activated thioester coenzyme A intermediates, and their functional impact on mitochondrial sirtuins. In a Perspective article, the role of mitochondrial dysfunction and the molecular mechanisms participating in necroptosis, a form of necrosis, are critically examined by Marshall and Baines (2014).

A relatively new area of research is covered by Dedkova and Blatter (2014) who extensively review the role of the ketone body β-hydroxybutyrate, its polymer poly-β-hydroxybutyrate, and inorganic polyphosphate, in diverse cellular functions, including mitochondrial ion transport, energetics and activation of mitochondrial permeability transition by polyphosphate.

Detailed mechanisms of potential cardioprotection by volatile anesthetics to target mitochondrial channels/transporters and ETC complexes are described in Agarwal et al. (2014). Recent emergent molecular processes mediating physical-metabolic interactions between lipid droplets and mitochondria and their potential impact on fatty acid oxidation and generation of signaling ROS are explored in the contribution by Aon et al. (2014).

Overall, the work in this Research Topic exemplifies many situations in which the morphological and functional behavior of mitochondria is sensitively tuned to the changing cellular energetic-redox status. The Research Topic sought clues about the underlying mechanisms that allow mitochondria to accomplish their energetic function while at the same time confronted with the unavoidable redox hurdle of processing huge amounts of oxygen as well as preserving the cellular redox environment. Since these conditions could be antithetical with the organelle's survival, many contributions shed light on the fact that mitochondrial targeted approaches to treat diseases could be a harbinger for their protection and concomitantly cytoprotection in different pathologies. In this case, we have come full circle: the protector, the mitochondrion, has to be protected.

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Reactive oxygen species and redox compartmentalization

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Reactive oxygen species (ROS) formation and signaling are of major importance and regulate a number of processes in physiological conditions. A disruption in redox status regulation, however, has been associated with numerous pathological conditions. In recent years it has become increasingly clear that oxidative and reductive modifications are confined in a spatio-temporal manner. This makes ROS signaling similar to that of Ca^{2+} or other second messengers. Some subcellular compartments are more oxidizing (such as lysosomes or peroxisomes) whereas others are more reducing (mitochondria, nuclei). Moreover, although more reducing, mitochondria are especially susceptible to oxidation, most likely due to the high number of exposed thiols present in that compartment. Recent advances in the development of redox probes allow specific measurement of defined ROS in different cellular compartments in intact living cells or organisms. The availability of these tools now allows simultaneous spatio-temporal measurements and correlation between ROS generation and organelle and/or cellular function. The study of ROS compartmentalization and microdomains will help elucidate their role in physiology and disease. Here we will examine redox probes currently available and how ROS generation may vary between subcellular compartments. Furthermore, we will discuss ROS compartmentalization in physiological and pathological conditions focusing our attention on mitochondria, since their vulnerability to oxidative stress is likely at the basis of several diseases.

Keywords: reactive oxygen species, compartmentalization, mitochondria, oxidative stress, redox signaling

INTRODUCTION

Reactive oxygen species (ROS) formation and redox signaling are well known to play a major role in physiology as well as in a variety of pathologies. For instance, in the heart, cardiomyocyte differentiation, and excitation-contraction coupling are under tight redox control (Burgoyne et al., 2012; Steinberg, 2013). On the other hand, cardiac pathologies, such as ischemia/reperfusion injury, heart failure, and arrhythmias can be prevented or blocked by inhibiting specific processes that result in ROS generation in several experimental models (Takimoto and Kass, 2007; Youn et al., 2013; Anderson et al., 2014; Kaludercic et al., 2014b). Thus, it appears that pro-oxidant generation and antioxidant defense need to be tightly regulated (Chance et al., 1979). Indeed, disruption of redox signaling and control, and imbalance in favor of pro-oxidant species is defined oxidative stress, term first coined in 1985 (Sies, 1985; Sies and Cadenas, 1985; Jones, 2006). Conversely from pathological modifications (Chance et al., 1979; Powers and Jackson, 2008), it appears that physiological redox signaling is characterized by reversible oxido-reductive modifications, confined both spatially and temporally in subcellular compartments and microdomains.

To exert their effects, ROS have to induce a reversible change that results in the modification of protein activity. The first step is the single-electron oxidation of a thiol to a thiyl radical, which can then react to form disulphide bonds with glutathione (GSH) or with another protein thiol (Wardman and Von Sonntag, 1995; Collins et al., 2012). Nevertheless, thiols can be further oxidized by ROS and result in higher oxidation states of sulfur (Steinberg, 2013). Such changes have limited or no reversibility under biological conditions (Jones and Go, 2010; Steinberg, 2013).

Quantification of thiol oxidation on cellular and subcellular levels has shown that thiol/disulphide couples such as GSH and thioredoxin (Trx) are maintained at stable values and are not in equilibrium relative to each other in different organelles (Go and Jones, 2008; Jones and Go, 2010). This suggests that redox status as a consequence of ROS production is not necessarily a global imbalance of oxidative and reductive processes, but rather that thiol oxidation in different cellular compartments serves as means for cell signaling, protein trafficking and regulation of enzyme, receptor, transporter and transcription factor activity (Balaban et al., 2005; D'Autreaux and Toledano, 2007). This consideration, termed the "redox hypothesis," postulates that oxidizable thiols are control elements organized in redox circuits that are physically and kinetically separated so that they are highly responsive and can function independently to regulate different biological processes (Jones and Go, 2010). However, upon a certain threshold in ROS formation, these circuits can be disrupted. Indeed, the occurrence of oxidative stress overwhelms the cellular antioxidant defense and results in lack of control over redox signaling mechanisms. These concepts can now be validated employing new redox sensors that allow dynamic and compartmentalized ROS

measurements and their correlation with organelle/cell function and viability.

Thus, ROS generation within specific subcellular compartments and their redox status appear to be of major importance for understanding cell pathophysiology. Recently, new methods for the study of redox compartmentalization have become available. This is a rapidly growing field that led to the development of, and was then contributed by, probes that now permit observation of rapid redox changes in real time and with single organelle resolution not only in live cells, but also in living animals (Woolley et al., 2013; Ezerina et al., 2014; Lukyanov and Belousov, 2014). Here we will review the tools currently available for the measurement of ROS and redox potential within single organelles and discuss the data available so far on ROS compartmentalization in physiological and pathological conditions focusing our attention on mitochondria as the major source and target of ROS.

TOOLS TO STUDY COMPARTMENT REDOX STATUS AND ROS FORMATION

In order to study the relationship between ROS formation and cell (dys)function, it is necessary to define which species are produced, in what amount and to characterize them in a spatio-temporal manner. Redox potential of a specific compartment or cell can be studied using a variety of techniques to identify and quantify major redox couples or redox sensitive proteins within organelles. For instance, high-performance liquid chromatography (HPLC) is used for the quantification of GSH/GSSG and NAD(P)H/NAD(P)$^+$ redox potentials (Jones, 2002; Takimoto et al., 2005), whereas mass spectrometry and redox Western blotting, in association with labeling of free thiols, are frequently used to determine the redox state of several proteins such as Trx, Trx reductase, and others (Halvey et al., 2005; Chen et al., 2006; Go et al., 2009; Go and Jones, 2013). Although these methods present high specificity for the redox couple examined and both the oxidized and reduced form can be quantified, they often require tissue/cell fractionation, during which redistribution and artifactual oxidation/reduction can occur. To overcome these problems molecular biology techniques using epitope-tagged versions of nuclear localization sequence (NLS)-Trx-1 and nuclear export sequence (NES)-Trx1 (specifically localized in nuclei or cytoplasm) have been developed that allow measurements without fractionation (Go et al., 2010).

More recent approaches involve fluorescent imaging techniques of ROS and major redox couples within organelles in intact cells or organisms *in vivo*. Currently available fluorescent sensors for compartmentalized ROS detection can be divided into small molecule probes and genetically encoded fluorescent proteins. The overview of the methods presented here is by no means exhaustive and for in depth coverage the reader is referred to the following excellent reviews (Meyer and Dick, 2010; Go and Jones, 2013; Lukyanov and Belousov, 2014; Winterbourn, 2014).

SMALL MOLECULE REDOX PROBES

Although several small molecule fluorescent probes are available, only a few of them can be targeted to specific subcellular compartments (**Table 1**). It should be also mentioned that, to some extent, all these probes present limitations in terms of selectivity and sensitivity.

MitoSOX Red is widely used for measurement of superoxide formation in the mitochondria of live cells (Robinson et al., 2006; Zhou et al., 2011a). MitoSOX Red indicator is a derivative of hydroethidine (HE) and contains the cationic triphenylphosphonium substituent that is responsible for the electrophoretically driven uptake of the probe in actively respiring mitochondria. The reaction between superoxide and HE generates a highly specific red fluorescent product, 2-hydroxyethidium. Nevertheless, another red fluorescent product, ethidium, can be formed from other oxidants in biological systems (Zhao et al., 2005). Thus, a simple fluorescence assay cannot distinguish between superoxide and other oxidants. The superoxide-specific product can be detected by HPLC or mass spectrometry and only then it provides a reliable method for superoxide production (Zhao et al., 2005; Zielonka et al., 2009).

Reduced MitoTracker dyes, MitoTracker Orange CM-H_2TMRos, and MitoTracker Red CM-H_2XRos, are derivatives of dihydrotetramethyl rosamine and dihydro-X-rosamine, respectively. These reduced probes become fluorescent and positively charged upon their oxidation in live cells, and thus accumulate in mitochondria according to the Nernst equation (Poot et al., 1996; Kweon et al., 2001; Kaludercic et al., 2014a). As with MitoSOX Red, the quick and easy loading into the cells makes these probes very convenient. However, reduced MitoTracker dyes are not specific for single oxidant species and the fact that their

Table 1 | Small molecule fluorescent redox sensitive probes and their characteristics.

Redox sensor	Compartment	Excitation wavelength, nm	Emission wavelength, nm	Detected ROS	System used	References
MitoSOX Red	Mitochondria	(396)510	580	$O_2^{\bullet-}$	Intact cells	Robinson et al., 2006
MitoTracker Red CM-H_2XRos	Mitochondria	579	599	Not specific	Intact cells	Poot et al., 1996
MitoTracker Orange CM-H_2TMRos	Mitochondria	554	576	Not specific	Intact cells	Kweon et al., 2001
Peroxy Lucifer 1 (PL1)	Cytosol	410	475/540	H_2O_2	Intact cells	Srikun et al., 2008
Nuclear Peroxy Emerald 1 (NucPE1)	Nuclei	468/490	530	H_2O_2	Intact cells, *in vivo*	Dickinson et al., 2011
Mitochondrial Peroxy Yellow 1 (MitoPY1)	Mitochondria	510	528	H_2O_2	Intact cells	Dickinson et al., 2013
SHP-Mito	Mitochondria	342/383	470/545	H_2O_2	Intact cells	Masanta et al., 2012
MitoBoronic acid (MitoB)	Mitochondria	Mass spectroscopy		H_2O_2	*In vivo*	Cocheme et al., 2011; Logan et al., 2014

accumulation is dependent on the mitochondrial membrane potential may lead to artifactual measurements.

In order to overcome problems associated with oxidant sensitive dyes, new generation of fluorescent probes has been developed. These are often referred to as "non-redox" probes as they contain a masked fluorophore that is released by the attack of the oxidant on the blocking group, without changing the oxidation state of the fluorophore (Winterbourn, 2014). The boronate derivatives, i.e., sensors that have boronate as blocking group, have been synthesized for the detection of hydrogen peroxide (H_2O_2) (Miller et al., 2005). Nevertheless, it was shown that some boronate probes also respond to peroxynitrite and hypochlorous acid, thus raising some concerns regarding their specificity (Sikora et al., 2009). Peroxy Green1 (PG1) and Peroxy Crimson1 (PC1) are second-generation probes that are sensitive enough to report H_2O_2 production at physiological signaling levels while maintaining H_2O_2 specificity and are activated by a single boronate deprotection (Miller et al., 2007). Because of their enhanced turn-on responses to H_2O_2, these new chemical tools are capable of detecting endogenous bursts of H_2O_2 produced by growth factor signaling in living cells (Miller et al., 2007; Lin et al., 2013). Nevertheless, these probes were not targeted to a specific compartment. There is a wide range of compounds with different fluorophores (Dickinson et al., 2010) and adapted structures to enable targeting to mitochondria (Dickinson et al., 2013) and other compartments, such as nuclei and endoplasmic reticulum (ER) (Srikun et al., 2008; Dickinson et al., 2011; Woolley et al., 2012). In particular, combining boronate-phenol chemistry with mitochondria-targeting functional group, such as positively charged phosphonium moiety, led to generation of Mitochondrial Peroxy Yellow (MitoPY1), SHP-Mito, and MitoBoronic acid (MitoB) (Cocheme et al., 2012; Masanta et al., 2012; Dickinson et al., 2013). SHP-Mito is also a ratiometric probe and allows for increased penetration depth and prolonged imaging time using two-photon microscopy (Masanta et al., 2012). MitoB instead is a ratiometric mass spectrometry probe that is rapidly converted to phenol product MitoP upon H_2O_2 oxidation (Cocheme et al., 2011). Measurement of MitoB/MitoP ratio has been successfully used *in vivo* and allows accurate measurements of H_2O_2 in the nanomolar range (Cocheme et al., 2011; Logan et al., 2014).

FLUORESCENT PROTEIN BASED REDOX SENSORS

Due to the need to overcome problems and limitations of conventional redox measurements related to specificity, reversibility, quantitation, and subcellular targeting, genetically encoded redox sensitive probes based on fluorescent proteins were developed (**Table 2**). This represents a major breakthrough, since these probes present high redox species specificity, their oxidation is reversible thus allowing dynamic real-time measurements and, importantly, can be targeted to specific subcellular compartments.

Initially, green fluorescent protein (GFP)-based redox sensitive proteins were developed introducing cysteine residues onto fluorescent protein scaffolds (Ostergaard et al., 2001). The redox state of these cysteines equilibrates with the GSH/GSSG ratio in a process catalyzed by the thiol-disulfide exchanging enzyme glutaredoxin (Grx), and leads to changes in chromophore spectra upon oxidation or reduction. Indeed, the redox sensitive yellow fluorescent protein (rxYFP) allows for non-invasive quantitative imaging of the dithiol-disulfide equilibrium (Ostergaard et al., 2001, 2004; Hu et al., 2008; Banach-Latapy et al., 2013). However, rxYFP is an intensiometric rather than a ratiometric probe. Moreover, its equilibration in different redox states depends on Grx availability and is very slow, thus representing a rate-limiting factor. This limitation was overcome fusing rxYFP to Grx1 (rxYFP-Grx1), rendering it independent of host organism Grx availability (Bjornberg et al., 2006).

Introduction of cysteines into fluorescent proteins led to the generation of redox sensitive GFP (roGFP). Initially, roGFP1 was developed that provided ratiometric fluorescence readout and increased sensitivity compared to rxYFP (Dooley et al., 2004; Hanson et al., 2004). Among the 6 roGFP variants available at present, roGFP2 has the highest dynamic range and has been best characterized (Meyer and Dick, 2010). Moreover, imaging of roGFP2 is easier, since the anionic form of the chromophore (presenting stronger fluorescence) decreases and the protonated form (initially showing lower fluorescence) increases upon oxidation, whereas the opposite is true for roGFP1 (Lukyanov and Belousov, 2014).

Generation of a redox relay between the redox sensing domain and redox sensitive fluorescent protein provided both specificity and efficiency to the redox sensing process. This idea was further used to improve roGFPs available. roGFP2 was fused to Grx1 (Grx1-roGFP2) and responds to either GSH or GSSG in a time scale of minutes and senses redox potential changes between −240 and −320 mV (Gutscher et al., 2008). When incubated with H_2O_2, Grx1-roGFP2 was insensitive to oxidation and only addition of GSH to the mixture led to the oxidation of the sensor, indicating its high specificity for the GSH redox status. It is important to note that, although roGFPs contain cysteine residues close to the chromophore, these cysteines do not present high redox reactivity, influencing the oxidation state of the probe only upon enzymatic oxidation. roGFPs can be targeted to several compartments of the cell, such as cytosol, mitochondria, intermembrane space (IMS), ER, nucleus, lysosomes, and endosomes (Dooley et al., 2004; Waypa et al., 2010; Albrecht et al., 2011; Van Lith et al., 2011; Birk et al., 2013), or fused to specific proteins to immobilize it in the cell (Pal et al., 2013). In terms of applicability, conventional redox sensing fluorescent proteins such as rxYFP and roGFPs are more suitable for the measurement of steady state redox conditions, since the kinetics of complete intracellular equilibration of the probe with the GSH system is slow and can take tens of minutes (Meyer and Dick, 2010). On the other hand, chimeric fusion proteins of redox active enzymes and redox sensitive fluorescent proteins facilitate rapid and complete equilibration with a defined cellular redox couple. Indeed, Grx1-roGFP2 is currently the probe of choice for dynamic measurements of GSH/GSSG ratio in subcellular compartments (mitochondria, IMS, cytosol) in a variety of experimental settings, from intact cells to animals *in vivo* (Gutscher et al., 2008; Albrecht et al., 2011; Kojer et al., 2012; Breckwoldt et al., 2014).

The same group also developed Orp1-roGFP2 by fusing roGFP2 with yeast peroxidase Orp1 (Gutscher et al., 2009).

Table 2 | Genetically encoded fluorescent protein based redox sensors and their characteristics.

Redox sensor	Compartment	Excitation wavelength, nm	Emission wavelength, nm	Detected ROS	System used	References
FLUORESCENT PROTEIN BASED REDOX SENSORS						
rxYFP	Nuclei, cytosol, mitochondrial matrix, IMS	512	527	GSH/GSSG	Intact cells	Ostergaard et al., 2004; Hu et al., 2008; Banach-Latapy et al., 2013
roGFP1	Mitochondria, IMS, cytosol, nuclei, ER	400/480	510	GSH/GSSG	Intact cells	Dooley et al., 2004; Hanson et al., 2004; Van Lith et al., 2011; Birk et al., 2013; Rodriguez-Rocha et al., 2013
roGFP2	Cytosol, mitochondria, ER, nuclei	400/495	515	GSH/GSSG	Intact cells, *in vivo*	Dooley et al., 2004; Guzman et al., 2010; Birk et al., 2013
cpYFP	Mitochondria	405/488	515	$O_2^{\bullet -}$	Intact cells, *in vivo*	Wang et al., 2008; Fang et al., 2011; Shen et al., 2014
REDOX SENSITIVE FLUORESCENT PROTEINS COUPLED TO REDOX ACTIVE ENZYMES						
Grx1-roGFP2	Cytosol, mitochondria, IMS, ER, nuclei	405/488	515	GSH/GSSG	Intact cells, *in vivo*	Gutscher et al., 2008; Albrecht et al., 2011; Kojer et al., 2012; Birk et al., 2013; Breckwoldt et al., 2014
Orp1-roGFP2	Mitochondria, cytosol	405/488	515	H_2O_2	Intact cells, *in vivo*	Albrecht et al., 2011
HyPer	Cytosol, mitochondria, IMS, ER, peroxisomes, nuclei	420/488	515	H_2O_2	Intact cells, *in vivo*	Malinouski et al., 2011; Mishina et al., 2011; Lukyanov and Belousov, 2014
rxYFP-Grx1		512	523	GSH/GSSG	*In vitro*	Bjornberg et al., 2006
REDOX SENSITIVE FLUORESCENT PROTEINS FUSED TO MEMBRANE PROTEINS						
EGFR-HyPer	Plasma membrane, endosomes	420/488	515	H_2O_2	Intact cells	Mishina et al., 2011
PDGFR-HyPer	Plasma membrane, endosomes	420/488	515	H_2O_2	Intact cells	Mishina et al., 2011
HyPer-TA	Cytoplasmic side of ER membrane	420/488	515	H_2O_2	Intact cells	Mishina et al., 2011
p47phox-roGFP2	Plasma membrane	400/495	515	H_2O_2	Intact cells, *in vivo*	Pal et al., 2013
DuoxA1-OxyFRET	Plasma membrane	435	535/480	H_2O_2	Intact cells	Enyedi et al., 2013
DuoxA1-PerFRET	Plasma membrane	435	535/480	H_2O_2	Intact cells	Enyedi et al., 2013
roGFP1-Lamp2a, roGFP1-Cnx, roGFP1-CD63, TrfR-roGFP1	Lysosomes, ER, endosomes	400/480	510	GSH/GSSG	Intact cells	Austin et al., 2005
FRET BASED REDOX SENSORS						
OxyFRET	Mitochondria, cytosol, plasma membrane	435	535/480	H_2O_2	Intact cells	Enyedi et al., 2013
PerFRET	Mitochondria, cytosol, plasma membrane	435	535/480	H_2O_2	Intact cells	Enyedi et al., 2013
HSP-FRET	Cytosol	430	470/535	Not specific	Intact cells	Waypa et al., 2006; Robin et al., 2007
Organic Hydroperoxide Sensor (OHSer)	Cytosol, nuclei	519	526	H_2O_2	Intact cells	Zhao et al., 2010, 2013
NADPH SENSORS						
Frex	Mitochondria, cytosol, nuclei	420/500	535	NADH	Intact cells	Zhao et al., 2011
Peredox	Cytosol, mitochondria	400	510	NADH	Intact cells	Hung et al., 2011

This resulted in an H_2O_2-sensitive probe that is pH insensitive, ratiometric, and reports submicromolar concentrations of H_2O_2. This sensor has been targeted to the cytosol or to the mitochondria and allows dynamic H_2O_2 measurements both *in vitro* and *in vivo* (Albrecht et al., 2011).

Another H_2O_2 sensitive sensor with similar characteristics is HyPer, developed inserting the circularly permuted YFP (cpYFP) into the regulatory domain of *E. coli* H_2O_2 sensing protein OxyR (Choi et al., 2001). HyPer demonstrates specificity and submicromolar affinity to H_2O_2, and can be targeted to the cytosol, mitochondria, IMS, ER, peroxisomes, or nuclei (Malinouski et al., 2011). As with roGFPs, the advantages of HyPer are reversibility (it can be reduced by cellular thiol-reducing systems), and the possibility to perform ratiometric measurements thus preventing imaging artifacts caused by object movement or differences in expression levels between cells or compartments (Lukyanov and Belousov, 2014). However, one concern that deserves attention is the influence of pH on HyPer fluorescence. The excitation fluorescence ratio is significantly shifted by pH (even by a small shift of 0.2 pH units) and could lead to artifactual results (Meyer and Dick, 2010). Thus, when using HyPer it is necessary to monitor pH changes in the same compartment [for example using pH-indicator SypHer, a mutated form of HyPer generated mutating one of the two H_2O_2-sensing cysteine residues of the OxyR domain (Poburko et al., 2011)]. Indeed, the same problem of pH sensitivity occurs with cpYFP, initially developed as a superoxide sensor targeted to the mitochondrial matrix (Wang et al., 2008), making it unclear whether the observed changes are due to superoxide or pH changes. Recently, two enhanced versions of HyPer have been developed, HyPer-2 and -3, with HyPer-3 showing an expanded dynamic range and higher brightness upon expression in cells while maintaining the oxidation and reduction rates fast (Markvicheva et al., 2011; Bilan et al., 2013). HyPer has also been targeted to the plasma membrane and specific loci by fusing it to the epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR) or to the tail anchor sequence of the protein tyrosine phosphatase 1B (PTP-1B) (Mishina et al., 2011). The same has been done also with the roGFP2 that has been fused to the NADPH oxidase (Nox) organizer protein p47phox (Pal et al., 2013) making it possible to readily detect H_2O_2 close to the source of its production.

Due to their recent development, limited information is available on fluorescence resonance energy transfer (FRET) based redox sensors. Initially, a linker containing two cysteine residues was placed between enhanced cyan and yellow fluorescent protein FRET pair, but showed a too small dynamic range of the probes to be used for imaging (Kolossov et al., 2008). Other novel FRET probes were recently developed (OxyFRET, PerFRET, HSP-FRET, rOHSer) (Waypa et al., 2006; Robin et al., 2007; Zhao et al., 2010, 2013; Enyedi et al., 2013), and targeted to mitochondria, cytoplasm, nuclei or plasma membrane or they were fused to the dual oxidase (Duox) activator DuoxA1 to achieve colocalization with Duox1 (Enyedi et al., 2013). Nevertheless, more studies are necessary to fully characterize these sensors. For instance, OxyFRET contains N- and C-terminal cysteine rich domains of Yap1 as H_2O_2 sensitive regions, which relies on the peroxidase function of Orp1. Since Orp1 is not present in mammalian cells, it remains to be elucidated which cellular redox couples or enzymes are actually responsible for the oxidation of the probe (Enyedi et al., 2013).

Finally, two genetically encoded sensors for NADH (Frex, Peredox) were developed placing circularly permuted fluorescent proteins (cpFPs) into a tandem dimer of bacterial Rex protein, capable of binding NADH (Hung et al., 2011; Zhao et al., 2011). Incorporation of cpFP into a linker between two Rex subunits results in Rex dimerization upon NADH binding and change in fluorescence. These sensors demonstrate highly specific affinity for NADH and do not respond to NADH analogs, including NADPH. While the Frex sensor is based on cpYFP and thus requires pH control (Zhao et al., 2011), Peredox is pH-stable, but intensiometric (Hung et al., 2011). Moreover, the extremely high sensitivity of Peredox to NADH precludes its use in the mitochondrial matrix where the $NADH/NAD^+$ ratio is high. Nevertheless, mitochondrial redox status can also be monitored measuring NADH/flavin ratio through their autofluorescence, an approach based on the pioneering work by Britton Chance in the 50's (Chance and Baltscheffsky, 1958; Chance and Jobsis, 1959). The great advantage of this approach is the rapid response to stimuli and its minimal invasiveness, which permit mitochondrial redox status monitoring not only in intact cells, but also in different organs *in vivo* (Chance et al., 1962; Mayevsky and Chance, 1973, 1982). Indeed, technological advances in this field led to the development of devices for the measurement of NADH/flavin ratio and tissue vitality also in patients (Mayevsky et al., 1996; Mayevsky and Rogatsky, 2007).

LIMITATIONS

One of the major issues in live cell imaging is phototoxicity, which occurs upon repeated exposure of fluorescently labeled cells to illumination. In their excited state, fluorescent molecules tend to react with molecular oxygen to produce free radicals that can damage subcellular components and compromise the entire cell. Phototoxicity depends on several variables: (1) photochemical properties of the fluorescent protein, (2) its concentration and subcellular localization, (3) the excitation intensity. Importantly, the total excitation light dose should be kept to minimum and it is preferable to use probes with longer wavelength excitation light, since excitation at a shorter wavelength is more damaging to cells because of increased efficiency of ROS production (Dailey et al., 2006). Thus, the imaging process requires optimization in order to find the right balance between image quality and light induced damage that may alter cell physiology (Dixit and Cyr, 2003).

Fluorescent proteins are generally not phototoxic to cells, due to the fact that their fluorophores are buried deep within a polypeptide envelope. Nevertheless, it is not possible to exclude that alterations in the cell physiology occur due to introduction of a ∼30 kDa protein as a fluorescent sensor or that the presence of a redox sensitive probe may interfere with the physiological redox signaling. In addition, tissue and cellular oxygen distribution may also play a role in ROS detection, since it is not homogeneous (Williams et al., 2012; Lloyd et al., 2014). These concerns need to be taken into account, and, when possible, appropriate controls should be performed.

COMPARTMENTALIZATION OF ROS FORMATION AND REDOX SIGNALS IN PHYSIOLOGY AND PATHOLOGY

In recent years it has become increasingly clear that oxidative and reductive modifications are confined in a spatio-temporal manner. This makes ROS signaling similar to that of Ca^{2+} (Rizzuto and Pozzan, 2006; Brasen et al., 2010; Petersen, 2014) or other second messengers, such as cyclic adenosine monophosphate (cAMP) (Stangherlin and Zaccolo, 2012). Some subcellular compartments are more oxidizing (such as ER, lysosomes, or peroxisomes) whereas others are more reducing (mitochondria, nuclei). Although more reducing, mitochondria are especially susceptible to oxidation, most likely due to the high number of exposed thiols present in that compartment (Jones and Go, 2010). Here we will examine how ROS generation may vary between subcellular compartments and determine beneficial effects or lead to pathology.

MITOCHONDRIA

Mitochondria are considered as the most redox-active compartment in the cell accounting for more than 90% of oxygen utilization. Although the vast majority of oxygen undergoes complete reduction to water at the level of cytochrome oxidase, partial reduction accompanied by ROS generation can occur as well (Boveris and Chance, 1973). Several other mitochondrial proteins, such $p66^{Shc}$ and monoamine oxidases (MAOs) among others, are prominent sites for ROS generation (Kaludercic et al., 2014b). Nevertheless, mitochondria are apparently very well equipped with antioxidant defense systems and are capable of maintaining a high degree of GSH reduction under normal conditions (Hanson et al., 2004; Jones and Go, 2010). This tight control of the mitochondrial redox status might be interpreted as a mechanism of protection, since a low rate of ROS generation is a normal process in mitochondria whereas excess can lead to cell death. Indeed, mitochondria are very susceptible to oxidation and considered to be both the source and target of ROS. Moreover, an initial ROS burst from the mitochondria may trigger a process termed as "ROS-induced ROS release" from neighboring mitochondria, amplifying oxidative stress, and leading to cell death (Zorov et al., 2000).

Mitochondria play a key role in energy metabolism and thus, depending on the cell and tissue type, may act as nutrient and oxygen sensors. Ca^{2+} uptake by mitochondria tightly regulates cellular metabolism by stimulating the activity of several key dehydrogenases (Denton, 2009). The finely tuned interplay between mitochondrial ROS, Ca^{2+}, and glucose appears to be the regulatory mechanism for insulin release from pancreatic β-cells (Leloup et al., 2011; Maechler, 2013). Indeed, insulin release is stimulated by mitochondrial ROS in response to glucose and requires extracellular Ca^{2+} for this mobilization (Leloup et al., 2009). Moreover, ROS can promote insulin sensitivity through phosphatase and tensin homolog (PTEN) inactivation and phosphoinositide 3-kinase (PI3K)/Akt signaling (Loh et al., 2009). On the other hand, mitochondrial dysfunction, and enhanced ROS generation are associated with insulin resistance and thus, type 2 diabetes (Kim et al., 2008; Anderson et al., 2009; Szendroedi et al., 2012).

A recent study examining the fine line between mitochondrial redox signals in physiology and pathology was performed on mice expressing Grx1-roGFP2 sensor in neuronal mitochondria (Breckwoldt et al., 2014). A multiparametric *in vivo* imaging approach was used to assess mitochondrial function simultaneously measuring mitochondrial redox status, membrane potential, pH, and Ca^{2+} levels. Redox potential of axonal mitochondria was tightly regulated under physiological conditions, although individual mitochondria showed short-lived redox bursts followed by spontaneous and reversible changes in shape (contractions), rapid mitochondrial depolarization, and increase in pH. These changes were independent of mitochondrial Ca^{2+} levels, but highly dependent on mitochondrial ROS formation and were increased exposing axons to higher neuronal activity. This suggests that these reversible mitochondrial redox changes might serve as a signal to overcome acute challenges and protect from eventual damage. However, under pathological conditions, such as in a chronic amyotrophic lateral sclerosis model or axotomy, rapid mitochondrial Ca^{2+} increase led to long-lasting mitochondrial oxidation, irreversible changes in shape and opening of the permeability transition pore (PTP), suggesting that noxious levels of stress may induce more permanent, Ca^{2+}-induced, mitochondrial derangements.

Another study employing cpYFP expressed in cardiomyocyte mitochondria similarly showed that individual mitochondria undergo spontaneous bursts of superoxide production (termed superoxide flashes), triggered by transient openings of the mitochondrial PTP (Wang et al., 2008; Fang et al., 2011). These flashes increased in frequency after stress, such as anoxia/reoxygenation, and were generated by the respiratory chain. Moreover, flash frequency in early adulthood was recently shown to negatively correlate with lifespan in *C. elegans* (Shen et al., 2014). Of note, none of these studies performed an adequate pH control and thus remain controversial, since, as discussed above, cpYFP is very sensitive to pH. Therefore, changes in fluorescence could have been caused by transient alkalinization of the mitochondrial matrix (Schwarzlander et al., 2011, 2012). Indeed, pH sensor SypHer (representing an adequate pH control for cpYFP) was able to detect similar mitochondrial flashes (Quatresous et al., 2012; Santo-Domingo et al., 2013) along with increase in MitoSOX fluorescence, which is stable at physiological pH. Therefore, more studies are needed to elucidate this controversy, but at the moment it cannot be excluded that actually both processes (superoxide and pH bursts) may take place.

Similar bursts in mitochondrial ROS formation were correlated with oscillations in organelle membrane potential and cardiomyocyte Ca^{2+} spark frequency under both physiological and pathological conditions (Aon et al., 2003, 2007, 2008; Zhou et al., 2011a) and were observed also in yeast cells, driven by the ultradian clock (Lloyd et al., 2003). Indeed, it has been proposed that individual, weakly coupled oscillating mitochondria are present in physiological conditions when ROS levels are low, but when the cellular redox status is perturbed, the mitochondrial network throughout the cell locks to one main low-frequency, high-amplitude oscillatory mode (Aon et al., 2008; Kurz et al., 2010). Transient changes in mitochondrial redox status, membrane potential, and uncoupling were also observed during normal autonomous pacemaking in dopaminergic neurons in the substantia nigra pars compacta (Guzman et al., 2010).

Nevertheless, disruption of these processes compromised Ca^{2+}-induced uncoupling and increased oxidation of matrix proteins, providing an explanation for their loss in Parkinson's disease. These studies suggest that transient changes in mitochondrial ROS, membrane potential, and shape might be part of a protective mechanism by which a signal is produced within a single organelle to isolate it from the rest of the mitochondrial network prompting its selective removal through mitophagy.

Mitochondrial ROS formation may also act as a signal to regulate organelle homeostasis. Hypoxia-inducible factor 1α (HIF1α), activated by hypoxia, is suggested to respond to and modulate mitochondrial oxidant production, through transcriptional regulation of several microRNAs that control the expression of components of the electron transport chain, lactate dehydrogenase A and PDK1 (pyruvate dehydrogenase kinase 1) (Guzy et al., 2005, 2008; Mansfield et al., 2005). Moreover, the increase of mitochondrial ROS needs to be conspicuous and diffuse to the cytosol in order to stabilize HIF1α suggesting that a certain threshold needs to be reached for this signaling to occur (Guzy et al., 2008). Increased mitochondrial ROS formation and HIF1α activation have also been proposed as the main mechanism involved in lifespan extension in *C. elegans* (Schulz et al., 2007; Lee et al., 2010b; Yang and Hekimi, 2010). Moreover, peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α), a transcription factor activated by mitochondrial ROS, HIF1α stabilization (O'Hagan et al., 2009), or through AMP-activated protein kinase (AMPK) stimulation (Canto et al., 2009), is required for the induction of many ROS-detoxifying enzymes, including GPx1 and superoxide dismutase 2 (SOD2), thus protecting the cell from oxidant induced death (St-Pierre et al., 2006).

A number of studies have evaluated ROS formation and redox status of the mitochondrial compartment in pathological conditions. Recent evidence points to mitochondrial oxidants as a signal for inflammasome activation (Bulua et al., 2011; Zhou et al., 2011b; Finkel, 2012). Moreover, oxidized mitochondrial redox state positively correlates with the metastatic potential and aggressiveness of breast cancer and melanoma (Li et al., 2009; Xu et al., 2010). Mitochondrial oxidative stress appears to precede ROS formation within other compartments and contributes to cell death during cardiomyocyte (Robin et al., 2007; Ranji et al., 2009; Loor et al., 2011), kidney (Hall et al., 2013) or liver ischemia (Haga et al., 2009) and vascular smooth muscle cells hypoxia (Desireddi et al., 2010; Waypa et al., 2010). Moreover, also other stimuli, such as parkinsonian toxins (Rodriguez-Rocha et al., 2013), metals (Hansen et al., 2006; Cheng et al., 2010) and nutrient deprivation (Go et al., 2007) can trigger mitochondrial oxidative stress. Tumor necrosis factor α (TNFα) also results in compartmentalized ROS formation in the mitochondria, Trx2 oxidation, downstream signaling to cytoplasm with nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activation and apoptosis (Hansen et al., 2004). In this study, cytosolic Trx1 was not oxidized, although more recent evidence employing mitochondria and cytosol targeted HyPer suggested that H_2O_2 increase was higher in the cytoplasm while mitochondrial matrix showed a lower response (Malinouski et al., 2011). Trx2 overexpression is protective against oxidant-induced apoptosis and several studies have shown that Trx2 is selectively susceptible to oxidative stress relative to cytoplasmic or nuclear Trx1 (Jones and Go, 2010), thus demonstrating the vulnerability of mitochondria to oxidative stress in a variety of cell types and pathologies (Kuroda et al., 2010; Stanley et al., 2011; Tocchetti et al., 2012).

Mitochondrial ROS formation is of major importance also in cardiovascular diseases (Stowe and Camara, 2009; Camara et al., 2010; Kaludercic et al., 2011, 2014b). Several sites in mitochondria have been shown to generate ROS and thereby contribute to cardiac damage, but very few of them can be targeted pharmacologically and are therefore not suitable for therapy. Nevertheless, MAOs represent a promising therapeutic target. MAOs are flavoenzymes localized in the outer mitochondrial membrane responsible for neurotransmitter and biogenic amine catabolism. Recent work demonstrated that both MAO-A and -B activation results in mitochondrial ROS generation that promotes pathological hypertrophy and heart failure *in vivo*, cardiomyocyte death and ischemia/reperfusion injury (Bianchi et al., 2005; Pchejetski et al., 2007; Kaludercic et al., 2010, 2014a; Villeneuve et al., 2013). Inhibition of these enzymatic activities *in vivo* maintained cardiac function in pressure overloaded mice and prevented the transition to heart failure demonstrating once again the importance of mitochondrial ROS generation and its implication in pathology. Importantly, we demonstrated the existence of a direct link between MAO activation, mitochondrial ROS formation, and mitochondrial dysfunction (Kaludercic et al., 2014a). Using the H_2O_2 sensor HyPer targeted specifically to mitochondria or cytosol, ROS formation following MAO activation was analyzed in a spatio-temporal manner (**Figure 1**). We observed that H_2O_2 formation occurs much earlier at the mitochondrial level compared to the cytosol and was independent of pH, since SypHer fluorescence ratio remained unchanged under the same conditions. These redox changes were followed by the loss of mitochondrial membrane potential. This is an important finding, since it reiterates the issue that mitochondria are "early targets" of endogenously produced oxidative stress that leads to mitochondrial dysfunction. On the other hand, mitochondrial ROS can also trigger the activation of signaling cascades and transcription factors in other compartments, such as cytosol (as described above). In addition, mitochondrial ROS formation through MAOs was shown to lead to the oxidation of myofibrillar proteins in the failing heart, an event that negatively correlated with cardiac function (Canton et al., 2006, 2011).

CYTOPLASM

Besides being affected by mitochondrial ROS formation, physiological stimulation at the plasma membrane can cause the oxidation of specific redox couples in the cytosol without affecting other subcellular compartments. This is the case of EGFR activation that is accompanied by ROS formation, a critical component for proper signal transduction (Mishina et al., 2011). Indeed, EGFR activation led to the oxidation of cytoplasmic Trx1 redox potential by nearly 20 mV while nuclear and mitochondrial Trx2 and cellular GSH were not affected at all (Halvey et al., 2005).

Redox couples in the cytoplasm are not necessarily in equilibrium and the biggest advantage of this is the achievement of distinct signaling effects. Indeed, Trx1 and GSH/GSSG couples

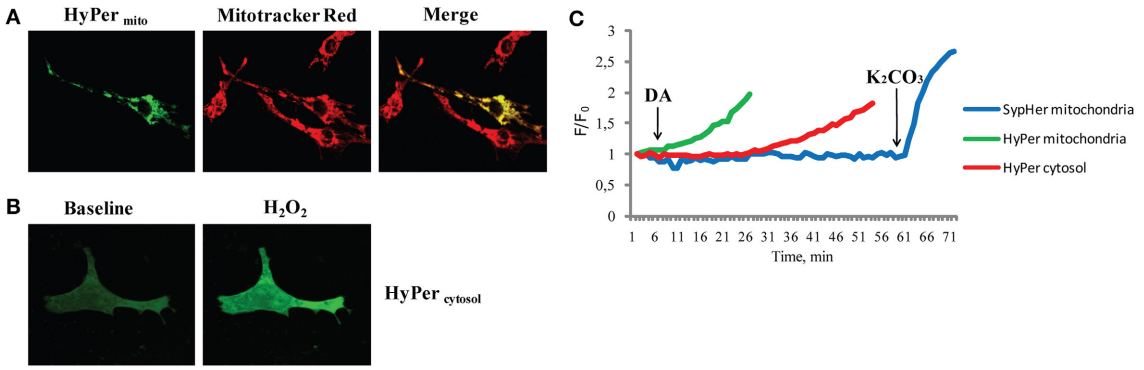

FIGURE 1 | H_2O_2 formation following MAO activation in different subcellular compartments. (A) Neonatal rat ventricular cardiomyocytes transfected with genetically encoded mitochondria targeted H_2O_2 sensor HyPer ($HyPer_{mito}$). Mitochondrial localization is confirmed by colocalization with the mitochondrial dye Mitotracker Red. **(B)** Neonatal cardiomyocytes expressing cytosol targeted HyPer ($HyPer_{cytosol}$), at baseline and after the addition of H_2O_2 (100 μM). **(C)** The fluorescence ratio of mitochondria targeted HyPer (green line) increased shortly after the addition of MAO substrate dopamine (DA), wheras the cytosolic HyPer (red line) became oxidized only after prolonged incubation. In a separate experiment under the same conditions fluorescence of mitochondria targeted pH sensor SypHer (blue line) remained unchanged upon the addition of DA and increased only after matrix alkalinization with K_2CO_3. Reprinted by permission from Kaludercic et al. (2014a).

were found to vary independently during growth transitions, redox signaling, and metal induced toxicity (Go and Jones, 2008). Moreover, some studies reported that changes in GSH and H_2O_2 levels are highly restricted, not necessarily coupled and concurrent and H_2O_2 was found to positively correlate with life span in *D. melanogaster* (Albrecht et al., 2011). Others instead support the idea of pro-oxidative changes in association with aging and reduction in life span (Rebrin et al., 2004; Cocheme et al., 2011). These observations suggest that age dependent pro-oxidative changes are highly variable, restricted to particular tissues and underline the need to measure different oxidant species and redox couples separately and specifically in order to achieve complete biological information (Albrecht et al., 2011).

Oxidation occurring in the cytoplasmic compartment is frequently exploited as a signal that then triggers the translocation of proteins to other compartments to exert their function. One example of this mechanism is the redox dependent activation of transcription factors, such as NFκB, activator protein 1 (AP-1) and NF-E2-related factor 2 (Nrf2) (Sen and Baltimore, 1986; Devary et al., 1991; Hansen et al., 2004). NFκB activation occurs in the cytoplasm through IκB kinase mediated IκB phosphorylation, thus resulting in dissociation and release of NFκB for its translocation into the nucleus (Sen and Baltimore, 1986). Nox1 overexpression led to an increased antioxidant response element (ARE) reporter gene expression mediated by H_2O_2-dependent c-Jun N terminal kinase (JNK) and extracellular signal-regulated kinases (ERK1/2) activation in the cytosol, but without affecting cytoplasmic GSH and Trx1 redox state (Go et al., 2004). This is likely due to the formation of localized increases in ROS formation following Nox activation that are not necessarily reflected globally in the whole cytoplasm (see below "ROS microdomains" section).

Thus, an increase in intracellular ROS as a result of exposure to a number of different stimuli can lead to oxidation of cysteine residues in cytoplasmic proteins, such as kinases and phosphatases, ultimately affecting signal transduction processes (Cumming et al., 2004). Indeed, one may envision cytoplasm as a "buffer zone" that allows for a low background of ROS used for sensitive and specific signaling (D'Autreaux and Toledano, 2007; Go and Jones, 2008). However, depending on the nature and duration of the insult, oxidation in the cytoplasmic compartment can also lead to cell death. For instance, Trx1 downregulation or oxidation results in AMPK oxidation and inactivation and apoptosis signal-regulating kinase 1 (ASK-1) activation, respectively, eventually leading to cell death (Liu et al., 2000; Shao et al., 2014).

NUCLEI

Nuclear redox couples (GSH, Trx1) are maintained at more reduced values than the ones in the cytoplasm, protecting the genome from ROS-induced damage. Indeed, many studies found oxidation in mitochondria and cytoplasm, but were unable to detect any changes in nuclei exposed to oxidants (Jones and Go, 2010). Nuclei contain GSH, which is critical for nuclear cysteine containing proteins. Trx1 translocates to the nucleus from cytoplasm in response to a variety of stimuli and its redox state then remains distinct from the one in the cytoplasm (Go and Jones, 2008). GSH is important for the regulation of nuclear matrix organization, maintenance of cysteine residues on zinc-finger DNA binding motifs in a reduced and functional state, chromosome consolidation, DNA synthesis, DNA protection from oxidative stress and protection of DNA-binding proteins (Go and Jones, 2008). Indeed, several transcription factors, including AP-1, NF-κB, Nrf2, p53, and glucocorticoid receptor, contain a critical cysteine in the DNA binding region that is required for DNA binding (Go and Jones, 2008). These cysteine residues need to be in a reduced state in order to bind DNA and excessive ROS formation inhibits transcription factor—DNA binding (Abate et al., 1990; Toledano and Leonard, 1991; Hainaut and Milner, 1993; Bloom et al., 2002). Therefore, transcription factors can be activated in the cytoplasm through oxidation, but once in the nucleus their critical cysteine residues need to be reduced.

OTHER COMPARTMENTS

Oxidizing environment in the ER lumen, required for introduction of structural disulfides during protein folding and secretion, is generated through continuous formation of GSSG in oxidative protein folding (Csala et al., 2006). The GSSG is kept within the ER compartment, since the ER membrane is impermeable to GSSG. Central redox proteins responsible for oxidative protein folding are Ero1p and protein disulfide isomerase (PDI). ER also contains a reductase system regulated by GSH and used to reduce incorrect protein disulfides (Chakravarthi et al., 2006). It is currently assumed that the redox state of the ER is optimally balanced for formation of disulfide bonds using GSH as the main redox buffer, and that unfolded protein response following induction of ER stress causes a reduction of this organelle whereby the release of misfolded protein is prevented (Enyedi et al., 2010; Delic et al., 2012; Birk et al., 2013). Indeed, this is the case with thapsigargin, although tunicamycin (another ER stress inducer) did not induce any reduction in ER of the HeLa cells (Birk et al., 2013). Therefore, the relationship between ER stress and its redox status warrants further investigation.

Peroxisomes are ubiquitous organelles involved in lipid metabolism and contain a number of enzymes that generate ROS and NO. Catalase is the major peroxisomal enzyme responsible for H_2O_2 metabolism and it appears that under physiological conditions H_2O_2 diffusion is prevented through its rapid conversion to O_2. Interestingly, catalase expression is virtually absent in the peroxisomes of insulin-producing cells (Elsner et al., 2011). This lack of antioxidant defense impedes inactivation of peroxisome-generated H_2O_2 following fatty acid metabolism, thereby increasing the vulnerability of pancreatic β-cells to ROS-mediated lipotoxicity (Elsner et al., 2011). The importance of catalase in defense from oxidative damage is further supported by the beneficial effects afforded by targeting catalase expression in mitochondria or peroxisomes (Schriner et al., 2005; Dai et al., 2009, 2010; Lee et al., 2010a).

Both endosomes and lysosomes require reduction of disulfides for their function. Interestingly, a study measuring redox potentials of endocytic compartments by expressing roGFP fused to various endocytic proteins found that recycling endosomes, late endosomes, and lysosomes are oxidizing compartments, mimicking conditions in the ER (Austin et al., 2005). It could not be excluded that a minor subset of lysosomes could be reducing or that there could be subregions of reducing potential, implying that this topic deserves further investigation.

Finally, the extracellular redox state also plays an important role in the redox homeostasis. The GSH/GSSG, Trx1, and Cys/Cyss couples are relatively oxidized in extracellular space as compared to the cytoplasm, the latter being quantitatively the most significant redox couple in the extracellular space (Go and Jones, 2008; Banerjee, 2012). Indeed, extracellular Cys/CySS redox couple oxidation or reduction differently regulates cell growth, an effect that is cell type dependent and mediated by intracellular kinase activation (Nkabyo et al., 2005; Ramirez et al., 2007). Plasma redox potentials are oxidized in association with age, chemotherapy, diabetes, cardiovascular disease, and smoking (Go and Jones, 2008; Banerjee, 2012; Menazza et al., 2014). Moreover, extracellular redox status can also sensitize cells to oxidant-induced apoptosis through mitochondrial compartment (Jiang et al., 2005) and/or induce intracellular signal transduction (**Figure 2**). For instance, an increase in oxidized Cys/CySS redox status in the extracellular space can trigger mitochondrial ROS formation mediated by redox potential sensitive plasma membrane and cytoskeletal proteins involved in inflammation (Go et al., 2010). Oxidized cysteine redox potential has also been shown to increase the secretion of the pro-inflammatory interleukin 1β (Iyer et al., 2009), suggesting that pro-inflammatory effects of oxidized plasma redox couples might be due to a mitochondrial signaling pathway (Go et al., 2010). In addition, oxidizing extracellular environment may lead to mitochondrial ROS formation that in turn activates Nrf2 to up-regulate antioxidant and detoxification systems, although the exact mechanism linking oxidizing extracellular conditions and mitochondrial ROS formation was not described (Imhoff and Hansen, 2009).

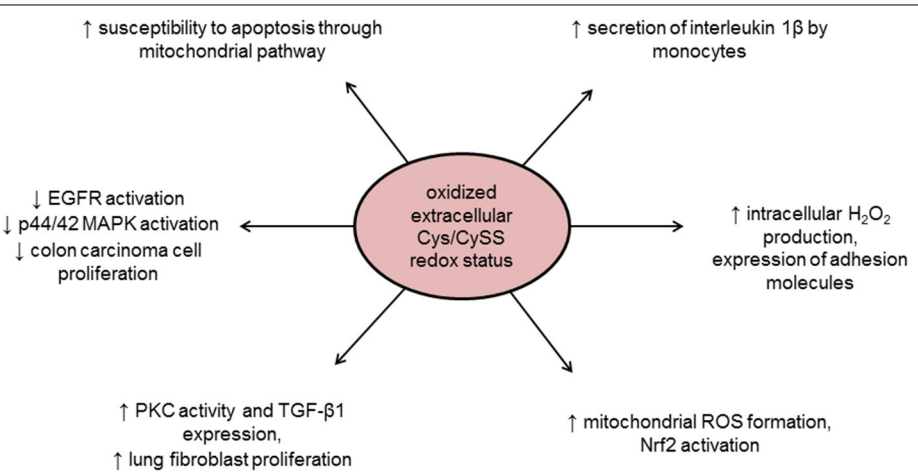

FIGURE 2 | Intracellular effects of oxidized extracellular redox conditions. Cys/CySS, cysteine/cystine ratio; EGFR, epidermal growth factor receptor; MAPK, Mitogen-activated protein kinase; Nrf2, NF-E2-related factor 2; PKC, protein kinase C; ROS, reactive oxygen species; TGF-β1, transforming growth factor beta 1.

ROS MICRODOMAINS

Redox status within the compartments can also be heterogeneous with localized areas of ROS production and others with more reducing environment. For instance, it is possible that when H_2O_2 is produced locally it only oxidizes a few redox sensor molecules and then diffuses so that the signal gets diluted in the cytosol. Fusion of HyPer to different proteins localized on the cytoplasmic face of the plasma membrane (EGFR, PDGFR), endosomes and the ER membrane allows visualization of sites of focal ROS formation within compartments (Lukyanov and Belousov, 2014). Activation of EGFR was associated with H_2O_2 microdomains (due to Nox activation) on the endosomes and the cytoplasmic side of the ER membrane, but no H_2O_2 formation was observed associated with the plasma membrane. PDGFR activation instead generated H_2O_2 microdomains at the plasma membrane and only after prolonged incubation on the endosomes, suggesting that a specific plasma membrane-residing Nox pool was activated (Mishina et al., 2011) (**Figure 3**). Importantly, the diffusion of H_2O_2 within the cytoplasm was restricted to less than a 1 μm radius.

Another mechanism of signal transduction linking ROS and tyrosine phosphorylation is mediated through regulation of phosphatase activity. PTPs (and other structurally related phosphatases, such as PTEN) contain cysteine residues critical for enzyme activity, whose oxidation leads to their reversible inactivation (Tonks, 2005; Ostman et al., 2011). The prerequisite for signaling is reversibility of this modification, however it is not known whether localized production of ROS can account for specific inactivation of one phosphatase (or a pool) over another. Fusion of HyPer to the tail anchor of PTP1B (HyPer-TA) resulted in the expression of the sensor on the cytoplasmic surface of the ER membrane (as is the case with endogenously expressed PTP1B) (Mishina et al., 2011). Indeed, following EGFR or PDGFR activation HyPer-TA became oxidized with a different temporal pattern than described above for EGFR- or PDGFR-HyPer, suggesting that localized formation of ROS in vicinity of PTP1B is necessary for its specific activation and agonist-induced signal transduction.

Whether microdomains of ROS formation can occur also in mitochondria, for instance, on the outer/inner leaflet of the outer mitochondrial membrane, IMS or in the matrix remains to be established. So far, only a few studies attempted to elucidate this concept. roGFPs or HyPer targeted to the mitochondrial matrix, IMS, and cytosol showed that exposure of the cells to paraquat or MPP^+ resulted in oxidation in the mitochondrial matrix that preceded the one in the cytosol, but did not lead to oxidative stress in the IMS (Rodriguez-Rocha et al., 2013). Rotenone instead, led to an early increase in H_2O_2 in the IMS and mitochondrial matrix that was later followed by the cytosolic compartment (Malinouski et al., 2011). Moreover, exposure of smooth muscle

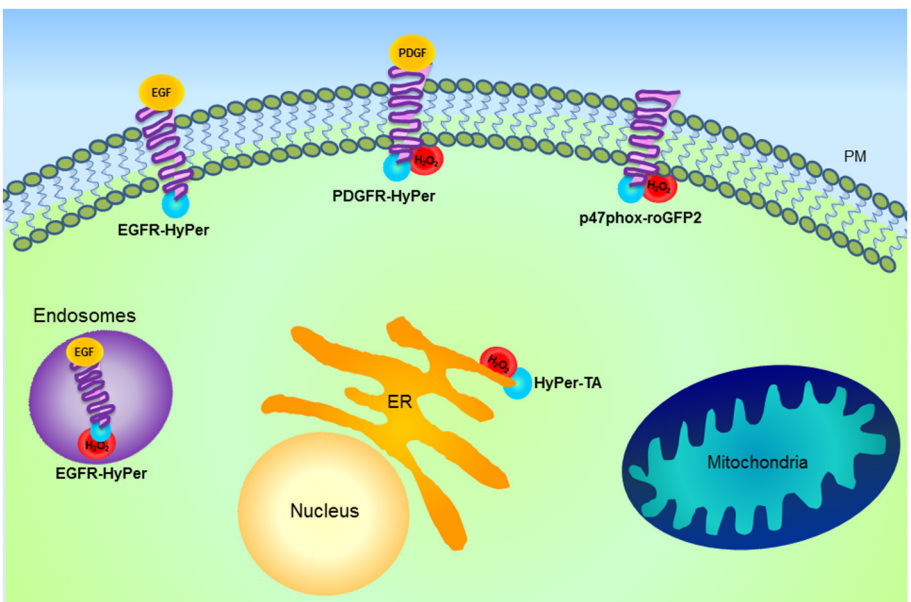

FIGURE 3 | Schematic representation of H_2O_2 microdomains within different subcellular compartments. Genetically encoded probe HyPer (blue) fused with different proteins including epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), Nox and tail anchor of protein tyrosine phosphatase 1B (HyPer-TA) is shown. HyPer fusion proteins allow the visualization of focal H_2O_2 formation. Activation of EGFR is associated with H_2O_2 microdomains formation (red) on the endosomes, while PDGFR activation generates H_2O_2 microdomains at the plasma membrane (PM) and only after prolonged incubation on the endosomes. Activation of both receptors results in the oxidation of HyPer-TA, localized at the cytoplasmic side of the endoplasmic reticulum (ER) membrane. This suggests that following agonist stimulation, there is specific activation of PM- or ER-associated Nox pools. Besides the indicated approaches for microdomain investigation, as detailed in the text, HyPer targeted to various cellular compartments has been used to characterize spatio-temporal differences in H_2O_2 formation. For instance, in the case of mitochondria information has been obtained by specific HyPer targeting to the matrix or intermembrane space, while microdomain characterization has not yet been exploited.

cells to hypoxia led to redox changes in the IMS and cytosol, but not in the mitochondrial matrix (Waypa et al., 2010). This suggests that areas of localized ROS formation or microdomains might exist within mitochondria, but further studies are necessary in this regard.

CONCLUSIONS

The available evidence shows that each compartment within the cell has different redox characteristics that are in line with the function of each organelle. ROS are generated in different compartments as part of normal metabolic function and may act as signaling molecules. However, depending on the intensity and duration, these redox signals can also become damaging, triggering, and participating in processes that lead to cell death. Although more reducing compared to other compartments, mitochondria present high rate of ROS formation and a number of ROS sources. Each compartment in the cell is characterized by redox signaling involved in a variety of biological processes, both physiological and pathological and it is likely that a cross-talk exists, by which ROS formation within one compartment can trigger their formation in another thereby amplifying overall oxidative stress. Development of novel tools to measure compartmentalized ROS formation and redox status displays several advantages: (1) it allows for better characterization of ROS signals in compartments and microdomains, and (2) provides an explanation for antioxidants failure in treating several pathologies. The unsuccessful outcome of several clinical trials evaluating the therapeutic potential of antioxidants for the treatment of heart failure raised questions regarding the importance of ROS or oxidative stress. Taking into account data available and given the importance of ROS both in physiology and pathology, we propose that inhibition of specific processes that generate ROS rather than general antioxidants administration may prove as a successful therapeutic strategy.

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The location of energetic compartments affects energetic communication in cardiomyocytes

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The heart relies on accurate regulation of mitochondrial energy supply to match energy demand. The main regulators are Ca^{2+} and feedback of ADP and P_i. Regulation via feedback has intrigued for decades. First, the heart exhibits a remarkable metabolic stability. Second, diffusion of ADP and other molecules is restricted specifically in heart and red muscle, where a fast feedback is needed the most. To explain the regulation by feedback, compartmentalization must be taken into account. Experiments and theoretical approaches suggest that cardiomyocyte energetic compartmentalization is elaborate with barriers obstructing diffusion in the cytosol and at the level of the mitochondrial outer membrane (MOM). A recent study suggests the barriers are organized in a lattice with dimensions in agreement with those of intracellular structures. Here, we discuss the possible location of these barriers. The more plausible scenario includes a barrier at the level of MOM. Much research has focused on how the permeability of MOM itself is regulated, and the importance of the creatine kinase system to facilitate energetic communication. We hypothesize that at least part of the diffusion restriction at the MOM level is not by MOM itself, but due to the close physical association between the sarcoplasmic reticulum (SR) and mitochondria. This will explain why animals with a disabled creatine kinase system exhibit rather mild phenotype modifications. Mitochondria are hubs of energetics, but also ROS production and signaling. The close association between SR and mitochondria may form a diffusion barrier to ADP added outside a permeabilized cardiomyocyte. But *in vivo*, it is the structural basis for the mitochondrial-SR coupling that is crucial for the regulation of mitochondrial Ca^{2+}-transients to regulate energetics, and for avoiding Ca^{2+}-overload and irreversible opening of the mitochondrial permeability transition pore.

Keywords: ADP, calcium, cardiomyocytes, creatine kinase, energetic compartments, mitochondria, oxidative phosphorylation, regulation

REGULATION OF MITOCHONDRIAL ENERGY PRODUCTION IN CARDIOMYOCYTES

The heart can never rest. During high workloads, skeletal muscle may develop an "energy debt," which is paid back during rest. But the heart must avoid such an energy debt, as it cannot take a few minutes off to rest and restore its energy levels. Therefore, it is crucial to quickly and precisely regulate energy generation to match the energy consumption in time and space. The primary energy source in the heart is mitochondrial oxidative phosphorylation, which is mainly regulated by Ca^{2+} and ADP/P_i-feedback.

Ca^{2+} exhibits "parallel regulation" of myofibrillar contraction and mitochondrial energy supply. Ca^{2+} enters the mitochondria through the Ca^{2+} uniporter, and is pumped out again mainly via the mitochondrial Na^+/Ca^{2+}-exchanger (Wei et al., 2011). This happens on a beat-to-beat basis. Mitochondrial Ca^{2+}-transients have the same time to peak as cytosolic Ca^{2+}-transients, but slower decay (Lu et al., 2013). Ca^{2+} in the mitochondrial matrix stimulates pyruvate, isocitrate and α-ketoglutarate dehydrogenase (McCormack et al., 1990), which reduce NAD to NADH in the citric acid cycle, as well as the F_1F_0-ATPase (Territo et al., 2000). Mitochondrial Ca^{2+}-uptake increases with contraction frequency and adrenergic stimulation (Lu et al., 2013). Thus, as the Ca^{2+}-transient increases to make cardiomyocytes contract faster and with greater force, so is the mitochondrial Ca^{2+}-uptake enhanced to further stimulate mitochondrial energy generation.

ADP and P_i, on the other hand, exhibit "feedback regulation" of mitochondrial energy supply. In contrast to Ca^{2+}, which has a steep electrochemical gradient and is let into the cytosol and pumped out, the feedback regulation depends on the energetic circuit, where ATP diffuses from the mitochondria to the ATPases, and ADP and P_i diffuse from the ATPases to the mitochondria. The importance of feedback as a regulator of mitochondrial energy supply is intriguing, because the heart exhibits a remarkable metabolic stability: the ADP-concentration is unchanged even during large increases in workload and oxygen consumption (Katz et al., 1989; Balaban, 2002). P_i-concentration changes the most, and some studies suggest P_i to be an important regulator, in particular in low to moderate workloads (Saks et al., 2000; Bose et al., 2003; Wu et al., 2008). Irrespectively, it is intriguing

that specifically in cardiomyocytes and red muscle, where feedback regulation is needed the most, there seems to be barriers that obstruct diffusion significantly.

In working heart trabeculae, NADH fluorescence decreases and then partially recovers upon an increase in work (Brandes and Bers, 2002). This suggests that there are multiple regulators of mitochondrial energy production. As noted above, Ca^{2+} stimulates dehydrogenases to produce NADH. It also stimulates F_1F_o-ATPase. But overall, Ca^{2+}-uptake by isolated mitochondria leads to an increase in NADH (Territo et al., 2001). NADH increases or decreases with P_i depending on the presence of ADP (Bose et al., 2003). ADP stimulates respiration rate, which decreases NADH fluorescence and increases flavoprotein fluorescence (Jepihhina et al., 2011). Cortassa and collaborators made an integrated model taking into account how excitation contraction coupling influences mitochondrial energetics (Cortassa et al., 2006). With this model, they were able to reproduce the experimental data of Brandes and Bers (2002) and analyze the regulatory mechanisms. Their quantitative analysis suggests that during work transitions energy supply is regulated initially by feedback, which decreases NADH. Subsequent parallel regulation by Ca^{2+} counterbalances this decrease, and NADH recovers (Cortassa et al., 2006). To explain how feedback can respond so quickly and be so important, despite overall metabolic stability, it is necessary to take into account energetic compartmentalization in cardiomyocytes.

It is well recognized that Ca^{2+} compartments exist in cardiomyocytes. Local Ca^{2+}-events are visible with Ca^{2+}-indicators (Wang et al., 2004). Due to the low Ca^{2+}-affinity of the mitochondrial Ca^{2+}-uniporter, mitochondrial Ca^{2+}-uptake would not take place if it were not for the structural proximity between mitochondria and the sarcoplasmic reticulum (SR) (Franzini-Armstrong, 2007). There is, however, no direct coupling, and Ca^{2+} has to diffuse from the SR to the mitochondria (Franzini-Armstrong, 2007). This leads to an intra-mitochondrial Ca^{2+}-gradient (Lu et al., 2013). The other major signaling molecule in the cell, cyclic AMP, is also confined to compartments. This is the only way to explain that stimulation of specific receptors using the same signaling cascade components (G_s proteins, cyclic AMP, and protein kinase A) leads to specific responses (Kritzer et al., 2012; Mika et al., 2012).

With these considerations in mind, it is not so surprising that cardiomyocytes also have energetic compartments with local concentrations of ADP, P_i, and ATP. Actually, energetic compartmentalization of cardiac tissue was suggested already in 1970, where Gudbjarnason et al. showed that after induction of ischemia, cardiac contraction declines with the concentration of phosphocreatine (PCr), while overall ATP remains unchanged (Gudbjarnason et al., 1970). It is difficult to assess energetic compartments as there are no good fluorescent indicators for ADP, P_i, and ATP (as for Ca^{2+}). As an indirect measure, many studies of energetic compartmentalization have assessed and/or modeled mitochondrial function in permeabilized fibers and cardiomyocytes—traditionally during ADP- and ATP-titrations. Mitochondria in permeabilized cardiomyocytes are characterized by an apparent ADP-affinity that is much lower than that of isolated mitochondria. This is taken to indicate significant diffusion restriction between the medium outside the permeabilized cell and the adenine nucleotide translocase (ANT) in the mitochondrial inner membrane. Questions that are still being debated are: What causes the restriction of diffusion? And how does it affect energetic communication between ATPases and mitochondria via feedback?

The exact location of energetic compartments may differ from other molecules compartments, but the basic principles of compartment formation are the same. The concentration of a molecule in a given location depends on (1) the reaction rate and relative location of proteins involved in its production/release and consumption/uptake, (2) its diffusion speed, which in turn depends (among other things) on its concentration gradient, (3) its buffering by particulate and/or soluble proteins in the cell, and (4) the organization of physical barriers in the form of membrane structures, organelles, and macromolecular complexes, which may obstruct diffusion. If the sites of synthesis and consumption are close to each other, the molecule may be immediately consumed thus not being able to diffuse to other parts of the cell. Thus, the molecule concentration will be locally much higher compared to the bulk, and the enzymes processing the molecule are said to be coupled. This seems to be the mechanism regulating the compartmentalization of cyclic AMP (Kritzer et al., 2012; Mika et al., 2012). Any enzyme pair with common substrate/product can become coupled. One prerequisite is, however, that they are bound in each other's vicinity, so that the substrate/product is channeled between the enzymes within the unstirred layer immediately above the surface (Goldman and Katchalski, 1971; Arrio-Dupont et al., 1985; Fossel and Hoefeler, 1987; Arrio-Dupont, 1988). If the molecule is consumed further away from its generation site, it has enough time to diffuse. Then, its distribution in the cell depends more on diffusion speed, buffering, and physical structures forming diffusion barriers. Much of molecular motion in the cell occurs by diffusion (Kinsey et al., 2011). For example, diffusion of ROS is effective in the micrometer range, making it a feasible mechanism of communication between mitochondria (Aon et al., 2004).

ENERGETIC COMPARTMENTS IN DIFFERENT SIZES

Experimental data suggest there to be multiple energetic compartments scaling in size from coupled enzymes to the proposed intracellular energetic units (ICEUs) (Saks et al., 2001). Starting with the smallest, good examples of coupled enzyme pairs are those of creatine kinase (CK), which is bound near and coupled to various cellular ATPases such as myosin ATPase (Ventura-Clapier et al., 1987; Arrio-Dupont, 1988; Haagensen et al., 2008), the sarco-endoplasmic reticulum Ca^{2+}-ATPase, i.e., SERCA (Minajeva et al., 1996), the Na^+/K^+ ATPase (Grosse et al., 1980), and the K_{ATP}-channel (Crawford et al., 2002). Here, the ATPases hydrolyze ATP to ADP and P_i, and CK uses PCr to regenerate ADP to ATP. In the mitochondria, the reaction goes the other way: ADP is phosphorylated to ATP, and the mitochondrial form of CK (Mi-CK) in the inter-membrane space uses creatine to regenerate ADP (Wallimann et al., 1992). Structural and model studies have confirmed that Mi-CK is bound near the ANT via its binding to cardiolipin in the inner mitochondrial membrane (Rojo et al., 1991; Schlattner et al., 2009;

Karo et al., 2012), suggesting direct metabolite transfer between them (Vendelin et al., 2004b). The reaction may also go in the direction of ADP and PCr synthesis in the cytosol, where CK may be coupled to glycolytic enzymes (Kraft et al., 2000).

Larger compartments depend more on physical structures forming diffusion barriers. At the next size level, compartments are represented by organelles. In cardiac tissue, mitochondria are the organelles taking up the largest volume, 20–30%, whereas next in size the SR represents 4.5% of the cell volume (Decker et al., 1991). In mitochondria, the double membrane results in an inter-membrane as well as a matrix compartment. In cardiomyocytes, there is a significant barrier for ADP at the level of the mitochondrial outer membrane (MOM). This was first suggested based on the apparent ADP-affinity being much lower in permeabilized fibers than in isolated mitochondria (Saks et al., 1991, 1993; Kuznetsov et al., 1996). Indeed, adenine nucleotides pass the MOM through the voltage gated anion channel (VDAC) (Rostovtseva and Colombini, 1997), and the permeability of VDAC can be regulated by tubulin (Rostovtseva et al., 2008; Rostovtseva and Bezrukov, 2012).

In cardiomyocytes, even larger compartments have been proposed to exist. They were named ICEUs (Saks et al., 2001). Although their delimiters are still not identified, it has been suggested that t-tubules, organelles, and macromolecular complexes are organized in such a manner that ATPases are grouped with mitochondria. The existence of ICEUs was proposed on the basis of experiments suggesting the existence of cytoplasmic diffusion restrictions. Diffusion restriction by MOM can explain the much lower ADP-affinity in permeabilized cardiomyocytes. However, it cannot explain the fact that ADP and P_i generated inside permeabilized cardiomyocytes seem to be "channeled" to the mitochondria rather than out of the solution (Kummel, 1988; Seppet et al., 2001). For this, there has to be cytosolic diffusion restrictions as well. Modeling shows that these are localized rather than uniformly distributed in the cytoplasm (Vendelin et al., 2004a). A more elaborate 3D model shows a possible arrangement of the diffusion restrictions, which are at the level of MOM and as sheets between mitochondria—probably formed by the SR and cytoskeletal proteins (Ramay and Vendelin, 2009).

ENERGETIC COMPARTMENTS AFFECT ENERGETIC COMMUNICATION—BUT HOW?

In the energetic circuit between ATPases and mitochondria, the main issue is how to efficiently transport ADP and P_i from ATPases to the mitochondria. This ensures an adequate phophorylation potential near ATPases and regulation of mitochondrial energy production. Overall, ADP ranges in the μM, whereas ATP, P_i, PCr, and creatine range in the mM (Wallimann et al., 1992). P_i-concentration varies the most with changes in work (Wu et al., 2008). However, due to its low concentration, even small changes in ADP have a large effect on the phosphorylation potential, which must be above a certain value for ATPases to obtain sufficient energy from ATP hydrolysis.

The physiological importance of coupled enzymes is quite obvious. The functional coupling between cytosolic CK and ATPases is beneficial when energy demand exceeds energy supply, and PCr is used to buffer the ADP/ATP-ratio and thus the phosphorylation potential. This situation has been dubbed "temporal energy buffering," that is characterized by a net consumption of PCr to buffer ATP. In cardiomyocytes, the functional coupling between Mi-CK and ANT is beneficial, because oxidative phosphorylation is the main source of energy, which can be stored as PCr. The CK equilibrium constant favors ADP phosphorylation. Thus, locally high ATP concentrations or direct transfer of ATP from ANT is needed for Mi-CK to generate PCr. The same is true for cytoplasmic CK coupled to glycolytic enzymes.

On a slightly larger scale, the situation becomes more debatable. In addition to the temporal energy buffering, CK has been suggested to function as a spatial buffer. The spatial buffering occurs because the CK system forms an energy circuit with creatine and PCr, which runs in parallel with that of ADP and ATP. In the presence of spatial energy buffering there is no net consumption of high-energy phosphates, i.e., consumption matches generation. Thus, the CK system or "CK shuttle" facilitates energetic communication between ATPases and ATP-producing sites.

In the heart that depends on reliable regulation of mitochondrial energy, the CK system has been assumed by other investigators to be paramount. Indeed, it seems paradoxical that in oxidative muscles, that rely on energy generated by mitochondria, there is a significant barrier obstructing the feedback from ATPases at the MOM level (Kuznetsov et al., 1996; Ventura-Clapier et al., 1998). As an explanation, it has been proposed that the MOM permeability is regulated to ensure energetic communication via the CK system (Saks et al., 1994). While this proposal is appealing, the role of the CK system continues to be debated. As noted above, it has been shown that dimeric tubulin binds VDAC and can restrict its permeability (Rostovtseva and Bezrukov, 2012). But it has yet to be established whether such a restriction actually occurs *in vivo* and whether this restriction is regulated in the heart. If such regulation occurs, we would expect MOM to be more permeable in the absence of a functional CK system.

Experiments where the CK system was inhibited by feeding with beta-guanidinoproionic acid (a creatine analog) or knockout of one or more CK isoforms have shown varying effects on cardiac function. In general, the hearts seem to adapt to cope with basal workloads, but they fail under high workload conditions, fast work transitions and ischemia (Shoubridge et al., 1985; Mekhfi et al., 1990; Zweier et al., 1991; Neubauer et al., 1999; Kaasik et al., 2001; Crozatier et al., 2002; Spindler et al., 2004; Nahrendorf et al., 2005). In studies of knockout mice, genetic background has turned out to be important, and backcrossing seems to result in a milder phenotype (Lygate et al., 2009, 2012). It has been surprising that, so far, no compensatory changes have been found in heart of mice lacking guanidineacetate methyltransferase (GAMT; an enzyme in the creatine synthesis pathway), where the CK system is non-functional due to lack of creatine. These mice are smaller in size (Schmidt et al., 2004) but exhibit the same exercise capacity and tolerance to chronic myocardial infarction of their wild type littermates (Lygate et al., 2013). Furthermore, in a follow up study it was shown that intracellular compartmentalization as well as diffusion across MOM and mitochondrial organization were unchanged (Branovets et al., 2013). GAMT deficient mice accumulate guanidinoacetate,

which is phosphorylated and may be used instead of creatine by CK in critical situations (Boehm et al., 1996; Kan et al., 2004). However, guanidinoacetate is not used by Mi-CK (Boehm et al., 1996), thus being unable to facilitate transport across MOM. Consequently, the cardiomyocytes of these mice do relatively well without a CK system, despite the fact that diffusion is restricted at the level of MOM to the same extent as in wildtype. This is in agreement with the idea that mitochondria are able to supply energy for SERCA-mediated Ca^{2+}-uptake with the same efficiency as CK (Kaasik et al., 2001). However, this interpretation raises the following question: is the CK system needed to facilitate ADP/ATP transport across MOM in the heart? At present, we have no firm answer to this question, and further studies are needed.

It must be noted that spatial energy buffering by CK may not exclusively mean that CK facilitates energetic communication across MOM. Experiments with mice overexpressing M-CK showed that the higher M-CK activity and the associated higher CK flux significantly improves cardiac function after ischemia-reperfusion (Akki et al., 2012) and in failing hearts (Gupta et al., 2012). Indeed, the decrease in CK flux can be used as a predictor of heart failure (Bottomley et al., 2013). Taken together these data indicate that lack of CK does not worsen heart failure and that M-CK overexpression has therapeutic potential (Lygate and Neubauer, 2014). Whereas the rescue by M-CK overexpression seems to be at odds with the lack of changes in knockout models, differences in metabolism and energy transfer during energy starvation might provide an explanation. In the healthy heart, CK activity is moderate and a significant fraction is accounted for by Mi-CK. In glycolytic muscles, the total CK activity is higher than in oxidative muscle, mainly due to cytosolic M-CK (Ventura-Clapier et al., 1998). Both ischemic and failing hearts exhibit insufficient mitochondrial energy generation, relying on glycolytic energy supply to a large extent. This prompts us to ask whether hearts overexpressing M-CK are rescued because of a higher M-CK activity enabling a more efficient energy transfer between glycolytic enzymes and ATPases.

The importance of CK as a spatial energy buffer has also been studied using theoretical approaches. Some of these studies support the idea that CK-mediated enhancement of the ADP feedback is important (Wu and Beard, 2009). A model based on NMR data suggested that under normal conditions, energetic communication may occur via direct ADP/ATP transport as well as creatine/PCr transport. In contrast, at high workload, the CK system is bypassed (Vendelin et al., 2010). A recent study shows that energy transport via the CK system amounts to no more than 15% (Hettling and van Beek, 2011). In fact, temporal and spatial energy buffering by the CK system are inseparable (Meyer et al., 1984). This is in agreement with the idea that the mitochondrial response to a change in heart rate is faster in CK knockout mice (Gustafson and van Beek, 2002). In view of these data, we ask whether in the heart, spatial energy buffering is needed under physiological conditions, and for facilitated transport across MOM.

Model simulations of kinetic data suggest moderate diffusion restriction at the MOM level, but not necessarily by MOM itself (Ramay and Vendelin, 2009; Sepp et al., 2010). Thus, a significant part of the low apparent ADP-affinity in permeabilized cells is due to cytoplasmic diffusion restrictions forming ICEUs. ICEUs explain why ADP is channeled to mitochondria rather than out of the cell (Seppet et al., 2001), and why mitochondrial ATP is as efficient as CK in providing ATP to SR and myofilaments with ATP (Kaasik et al., 2001). Hypothetically, ICEUs confine mitochondria and ATPases in smaller compartments to reduce diffusion distances and ensure direct energetic communication. However, it remains an open question whether the ICEUs are "designed" in the sense that some structures are specifically organized in order to form ICEUs, or whether ICEUs are simply the consequence of cellular organization.

Figure 1A shows the regular arrangement of mitochondria in a cardiomyocyte. **Figure 1B** displays the probability distribution of neighboring mitochondria around a central mitochondrion. Of note is the circular arrangement of mitochondria in the cross-section (Birkedal et al., 2006). Does this arrangement explain energetic coupling between mitochondria and ATPases? Rows of myofilaments are surrounded by rows of mitochondria that function as sinks for ADP and Pi. Oppositely, rows of mitochondria are also surrounded by rows of myofilaments that function as sinks for ATP. It seems natural that in a feedback system with energetic circuits, the majority of metabolites diffuse down their concentration gradients the shortest possible path rather than diffusing out of the permeabilized cell.

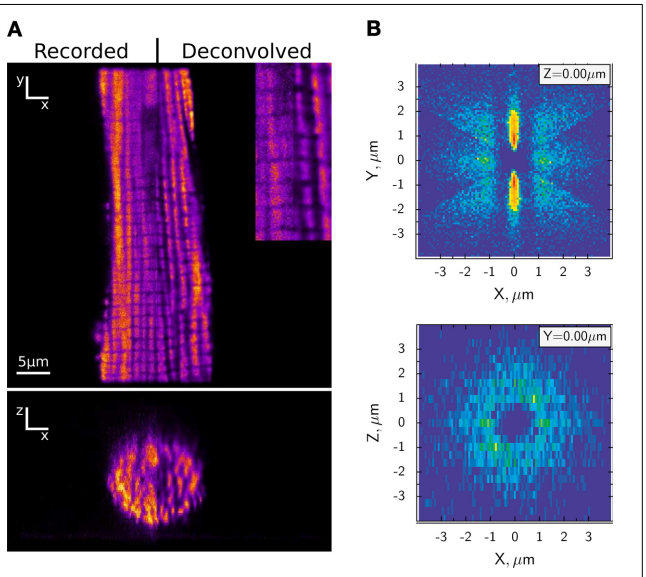

FIGURE 1 | Highly ordered arrangement of intermyofibrillar mitochondria in rat cardiomyocytes. (A) Representative confocal image of MitoTracker Green labeled mitochondria are shown on the top (XY) and reconstructed cross-section (XZ) at the bottom. The original images are compared with deconvolved images after applying algorithms developed in Laasmaa et al. (2011). **(B)** Probability density of the closest mitochondrial centers in each sector of a rat cardiomyocyte, calculated as described in Birkedal et al. (2006). The density is shown in pseudo color with blue corresponding to regions where no neighboring mitochondria were found and red to the regions with high probability of finding the center of neighboring mitochondria. Note that mitochondria are arranged in a regular pattern (XY plane) with parallel rows separated by ~1.8 μm that can be found in any transversal direction relative to each other (XZ plane). For details of the analysis, see Birkedal et al. (2006).

In the quest for the location of cytosolic diffusion restrictions, we have studied intracellular diffusion using raster image correlation spectroscopy (RICS). We first showed that radial diffusion of fluorescently labeled ATP in cardiomyocytes is slower than transversal diffusion (Vendelin and Birkedal, 2008). A later study used two fluorescent molecules of different size (Illaste et al., 2012). Here, it was intriguing to find that in the cardiomyocyte compared to solution, diffusion of the large molecule was less restricted than diffusion of the small molecule. A stochastic model predicted that diffusion restrictions form a lattice with dimensions that are in agreement with the cardiomyocyte ultrastructure (Illaste et al., 2012). In **Figure 2**, we take a step further and draw this lattice superimposed on the cardiomyocytes ultrastructure.

Two possible scenarios are represented in **Figure 2**. In the first one (**Figure 2A**), the lattice is superimposed onto the mitochondrial membranes and the m-band and z-line of the sarcomere. In the second one (**Figure 2B**), half of a mitochondrion is grouped together with a fraction of a sarcomere. Considering the structures in the cell, the former seems more plausible: Transversally, diffusion barriers are formed by mitochondrial and SR membranes whereas longitudinal barriers are constituted by protein-dense regions in the myofilaments (z-lines and m-bands) and probably with some help from the junctional SR and t-tubules. However, the problem is that diffusion restriction at the level of MOM seems to separate rather than group together mitochondrial energy generation and ATPase energy consumption. An alternative explanation would be that diffusional restrictions at MOM are not due to the membrane itself but a result of its close association with the SR. Much of the work on energetic compartmentalization uses permeabilized cardiomyocytes, which are kept in a relaxed state. Although non-physiological, this is a useful preparation since it represents a simple situation. However, the importance of the SR is difficult to study in permeabilized cardiomyocytes, as SERCA is not active in this preparation (Sepp et al., 2014). As a membranous structure, the SR associated with the mitochondria will restrict diffusion from the medium to mitochondria in permeabilized cardiomyocytes. However, it also forms the structural basis for the energetic coupling between SERCA and mitochondria (Kaasik et al., 2001).

A fully developed cardiac CK system with a relatively high expression of mitochondrial as well as cytosolic CK isoforms is mainly found in adult mammals. The fact that mitochondrial CK seems absent in the hearts of lower vertebrates such as fish and frog can be explained by that they have a lower body temperature and cardiac performance, and depend more on glycolytic energy production (Ventura-Clapier et al., 1998; Birkedal and Gesser, 2006; Sokolova et al., 2009). Likewise, neonatal mammals with lower cardiac performance and higher reliance on glycolysis do not express Mi-CK (Hoerter et al., 1991, 1994; Tiivel et al., 2000). As the cardiomyocytes mature, they increase in diameter and develop from a relatively simple morphology to multiple parallel rows of myofibrils and mitochondria organized in a crystal-like pattern as we know it in adult cardiomyocytes (Vendelin et al., 2005; Birkedal et al., 2006; Anmann et al., 2014). In parallel, they develop t-tubules and a more elaborate SR (Sedarat et al., 2000; Dan et al., 2007) as their excitation contraction coupling changes to depend less on trans sarcolemmal Ca^{2+}-transport and more on L-type Ca^{2+}-influx to trigger Ca^{2+}-release from the SR (Huang et al., 2008). During maturation the functional significance of cytosolic and Mi-CK increases (Hoerter et al., 1994). In light of the question whether CK facilitates transport across MOM, it is tempting to speculate that the increase in Mi-CK during development occurs simply because the cells transition from glycolytic to mitochondrial energy generation (Ostadal et al., 1999) and functional coupling of CK to an energy generation site is necessary for this reaction to happen in the direction of PCr synthesis. Probably, the concomitant decrease in apparent ADP-affinity is not for increasing energetic communication via the CK system, but due to the simultaneous development of the SR. The SR is less developed in lower vertebrates (Santer, 1985; Franzini-Armstrong and Boncompagni, 2011). In mammal heart, the SR develops significantly postnatally (Dan et al., 2007; Huang et al., 2008), and so does its association with mitochondria (Boncompagni et al., 2009). Thus, changes in SR-mitochondria interactions might provide an alternative explanation for the inter-species differences and developmental changes in the apparent ADP-affinity of permeabilized cardiomyocytes (Ventura-Clapier et al., 1998; Sokolova et al., 2009; Anmann et al., 2014). Additionally, the low

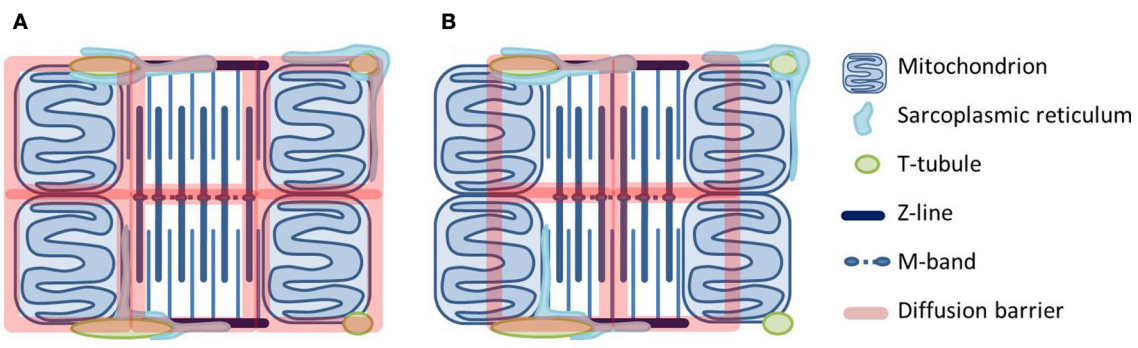

FIGURE 2 | Two scenarios for how diffusional barriers may be organized in cardiomyocytes. The schematic drawings are scaled according to Birkedal et al. (2006), Hayashi et al. (2009) and show mitochondria, t-tubules and sarcoplasmic reticulum (SR) around a sarcomere. The diffusional barriers are drawn to scale according to Illaste et al. (2012) and superimposed. In **(A)** the barriers are in agreement with the cell structures, but seem to separate mitochondria and myosin ATPases. In **(B)** mitochondria are grouped together with ATPases, but this scenario is difficult to explain in structural terms.

apparent ADP-affinity is specific for oxidative muscles (Kuznetsov et al., 1996; Ventura-Clapier et al., 1998). The difference between muscles might be explained by distinct expression of tubulin that regulate VDAC gating (Varikmaa et al., 2014). Another explanation involves SR-mitochondria interaction that seems to be more prominent in muscles that are rich in mitochondria (Franzini-Armstrong, 2007).

The close association of SR and mitochondria is crucial for proper function and energetic regulation. By ensuring mitochondrial Ca^{2+}-uptake, it allows for regulation of mitochondrial energy production by Ca^{2+} as well as ADP and P_i. Moreover, it ensures sufficient energy supply to SERCA. Indeed, mitochondria are as efficient as CK in providing energy for SERCA function (Kaasik et al., 2001). Furthermore, as is the subject of this special issue, mitochondria are not only energetic but also redox hubs since they generate reactive oxygen species (ROS) with signaling potential. Mitochondrial Ca^{2+}-uptake may take part in the regulation of ROS. As noted in the beginning, Ca^{2+} uptake by mitochondria increases NADH. Therefore, the Ca^{2+}-uptake under physiological conditions may have similar effects as the addition of respiratory substrates: increase in NAD(P)H in turn used to reduce glutathion and thioredoxin pools, important antioxidant systems modulating mitochondrial ROS-emission (Garcia et al., 2010; Stanley et al., 2011; Aon et al., 2012). Compromised energy provision affects SERCA activity and Ca^{2+}-re-uptake. This may lead to an increase in mitochondrial Ca^{2+}, which is known to be an important trigger of the permeability transition pore (mPTP) (Bernardi, 1999). mPTP opening is to some extent reversible. But high enough Ca^{2+} and stimulation by other factors leads to irreversible opening, and eventually to apoptosis and/or necrosis (Di Lisa et al., 2011). Functional coupling between SR and mitochondria will ensure energy supply to SERCA thus preventing excessive mitochondrial Ca^{2+}-uptake. In a situation of limited energy supply, transport of ADP and P_i from SERCA to the mitochondria may increase the mitochondrial Ca^{2+}-uptake capacity before mPTP opening is triggered (Wei et al., 2012; Sokolova et al., 2013).

CONCLUDING REMARKS

Feedback regulation of energetics depends on the location of diffusion barriers. We suggest an alternative explanation for the diffusion restriction at MOM level, namely that it is due to a close association of mitochondria and SR that ensures SERCA energy supply as well as mitochondrial regulation by Ca^{2+}. It has been suggested that in cardiomyocytes, the permeability of MOM itself is regulated by tubulin. Indeed, "closing the gates" to the mitochondria to enhance energetic communication by the CK system seems like a good explanation, if it is more efficient. There are, however, some studies suggesting that a significant fraction of the energetic communication can occur as direct transport of ATP, ADP, and Pi. Also, cardiac function is not severely compromised by the lack of a functional CK system although diffusion restriction at the level of MOM is unchanged.

Whether the compartmentalization of energy units control the feedback exerted by the CK system or merely exists as part of a structural organization to optimize energy transfer between mitochondria and ATPases via ADP, P_i, and Ca^{2+}, remain open questions.

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Cardiac mitochondria exhibit dynamic functional clustering

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Multi-oscillatory behavior of mitochondrial inner membrane potential $\Delta\Psi_m$ in self-organized cardiac mitochondrial networks can be triggered by metabolic or oxidative stress. Spatio-temporal analyses of cardiac mitochondrial networks have shown that mitochondria are heterogeneously organized in synchronously oscillating clusters in which the mean cluster frequency and size are inversely correlated, thus suggesting a modulation of cluster frequency through local inter-mitochondrial coupling. In this study, we propose a method to examine the mitochondrial network's topology through quantification of its dynamic local clustering coefficients. Individual mitochondrial $\Delta\Psi_m$ oscillation signals were identified for each cardiac myocyte and cross-correlated with all network mitochondria using previously described methods (Kurz et al., 2010a). Time-varying inter-mitochondrial connectivity, defined for mitochondria in the whole network whose signals are at least 90% correlated at any given time point, allowed considering functional local clustering coefficients. It is shown that mitochondrial clustering in isolated cardiac myocytes changes dynamically and is significantly higher than for random mitochondrial networks that are constructed using the Erdös–Rényi model based on the same sets of vertices. The network's time-averaged clustering coefficient for cardiac myocytes was found to be 0.500 ± 0.051 ($N = 9$) vs. 0.061 ± 0.020 for random networks, respectively. Our results demonstrate that cardiac mitochondria constitute a network with dynamically connected constituents whose topological organization is prone to clustering. Cluster partitioning in networks of coupled oscillators has been observed in scale-free and chaotic systems and is therefore in good agreement with previous models of cardiac mitochondrial networks.

Keywords: mitochondrial clustering, mitochondrial oscillator, functional connectivity, wavelets, cardiac myocyte

INTRODUCTION

The mitochondrial network in cardiac myocytes consists of highly organized and densely packed mitochondria whose inner membrane potential $\Delta\Psi_m$ can be triggered to enter several cycles of de-and re-polarizations by numerous stressors such as oxidative or metabolic stress (see Aon et al., 2008a for a review). These oscillations can be strictly localized in the form of transient single mitochondrial depolarizations (Nivala et al., 2011), individual or clustered mitochondrial $\Delta\Psi_m$ oscillations (Romashko et al., 1998; Kurz et al., 2010a) with clusters that can span the whole myocyte (Aon et al., 2004). Recruiting neighboring network mitochondria into an initial synchronized nucleus of a few mitochondrial oscillators has been described to be a fundamental process for global network synchronization (Strogatz, 2000, 2003; Aon et al., 2008b). During this process, a mitochondrial cluster can reach a critical size (sometimes referred to as "mitochondrial criticality" Aon et al., 2004, 2006) where mitochondria spontaneously self-synchronize, as in a phase transition. So far, investigations strongly support the fact that ROS-induced ROS release is a key player in such inter-mitochondrial communication or coupling (Zorov et al., 2006; Zhou et al., 2010; Nivala et al., 2011).

Recently, wavelet-based analysis tools have been developed to examine the mitochondrial network's spatio-temporal behavior under pathophysiological conditions (Kurz et al., 2010a,b); dynamic frequencies could be allocated to individual mitochondria and clusters of mitochondria with similar frequencies were identified that allowed for a quantitative characterization of the cluster's network properties. However, the network's topology (or connectivity properties) as opposed to its architectural organization (see Aon and Cortassa, 2012 for a review) has not yet been investigated quantitatively in terms of its clustering properties. Mitochondria in cardiac myocytes serve as the main energy supplier and modulator of the myocyte's mechanical and electrical processes, but are also modulated by the latter; therefore, the mitochondrial network's topological heterarchy becomes increasingly complex and non-linear (Yates, 1993). The functionality of an individual mitochondrial network node, though, can in part be characterized through its connectedness with other network nodes, (cf. Passingham et al., 2002), a result of the interplay of the entire complex mitochondrial network as an integrated system. The clustering coefficient can be used as a measure of the network's robustness toward the functional deletion of single mitochondria or

the network's efficiency to communicate (metabolic or other) information.

The present work investigates the presence of functional (dynamical) connectedness in the form of clustering of mitochondrial networks in isolated cardiac myocytes in comparison with clustering in random networks based on the Erdös–Rényi model. Functional network clustering is subsequently related to the network's spatio-temporal properties of the major cluster of mitochondria with similar frequencies.

MATERIALS AND METHODS

EXPERIMENTAL METHODS

All experiments were carried out on freshly isolated adult guinea pig ventricular myocytes at 37°C following protocols that were previously described (O'Rourke and Marban, 1994) with approval from the Johns Hopkins University Animal Care and Use Committee and in accordance with guidelines established in the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996).

In brief, cardiac myocytes were perfused with Tyrode solution (pH 7.5) containing 1 mM Ca^{2+} in the presence of 10 mM glucose and oscillations were triggered with a localized ($5 \times 5\,\mu m$) laser flash, as previously described (Aon et al., 2003). Mitochondrial inner membrane potential $\Delta\Psi_m$ was monitored with the cationic potentiometric fluorescent dye tetramethylrhodamine methyl ester (TMRE) and images were recorded with a two-photon laser-scanning microscope (MRC-1024MP, Bio-Rad) with excitation at 740 nm (Tsunami Ti:Sa laser, Spectra-Physics) and red emission of TMRE was collected at 605 nm using a band pass filter 578–630 nm (Aon et al., 2003).

SELECTION AND PROCESSING OF INDIVIDUAL MITOCHONDRIAL TMRE SIGNALS

As detailed before (Kurz et al., 2010a,b), TMRE signals of individual mitochondria were extracted from planar images of isolated cardiac myocytes recorded at a rate of dt by manually applying a grid template on a pixel-by-pixel basis to the averaged image of n subsequent images in time starting at the onset of TMRE oscillations. Parameter n was chosen such that $n \cdot dt$ was smaller or equal to the smallest period of all TMRE oscillations. Only myocytes with no shifts in the z-direction were included and shifts in the x and y-directions were corrected by moving the template grid accordingly.

The subsequent spatio-temporal signal analysis was conducted using the wavelet transform, correlation and coherence analysis as previously described in detail (Kurz et al., 2010a) and outlined below.

WAVELET ANALYSIS

With no prior knowledge of whether mitochondrial oscillations are stationary, wavelet analysis has been used to probe the dynamically changing frequencies of cardiac mitochondria (Kurz et al., 2010a). In this study, the same wavelet analysis was utilized: for each mitochondrion's TMRE signal, Morlet wavelets were taken where spacing between scales was set to $dj = 0.1$ and the smallest scale of the wavelets was chosen as $s_0 = 4dt$, signifying the smallest possible period for the detection of one oscillation. The total number of scales was determined as $j_1 = \log_2(N/s_0)/dj + 1$ with N being the total number of the recorded images per cell, thus resulting in scales that range from s_0 to $s_0 2^{(j_1-1)dj}$ and each scale having dj suboctaves. To determine cutoff frequencies, the longest period T of a synchronized oscillation of one cell was identified to determine the minimum cutoff frequency as $\nu_{min} = 1/1.1T$ and, similarly, the maximum cutoff frequency at $\nu_{max} = 1/s_0$. Power lineplots between ν_{min} and ν_{max} were interpolated for every scale with segments of 0.1 mHz and the maximum power for the interpolated plots was determined to eventually obtain maximal scale frequencies at each time point for each mitochondrion.

In **Figure 1A**, three random mitochondria labeled 1, 2, and 3 from an isolated cardiac myocyte are chosen to illustrate individual mitochondrial TMRE signal behavior. Evidently, only mitochondria 1 and 3 show marked oscillatory behavior over time whereas mitochondrion 2 is non-oscillating (see **Figures 1B,C**). **Figure 1C** shows the absolute squared wavelet transform of mitochondrion 3 over frequency and time. It can be seen that the main frequency component, depicted by the dark red color, varies between 15 and 20 mHz during the recording. This frequency component corresponds to the time interval between oscillation peaks and troughs observed in the upper panel of **Figure 1C**, that is approximately between 50 and 65 s.

SELECTION OF MITOCHONDRIA BELONGING TO A MAJOR CLUSTER

By obtaining frequency histograms of all mitochondria for each time point t, the maximum peak of each histogram was identified and other peaks were considered significant if their histogram amplitude was above 10% of the respective maximum peak (see also Figure 2A in Kurz et al., 2010a). If the mean TMRE signal of all mitochondria corresponding to the maximum peak had a cross correlation of 95% or higher with the mean TMRE signal of mitochondria in an adjacent peak, the latter was incorporated in the maximum peak and the procedure repeated with the next adjacent peak until the correlation dropped below 95%. The respective signal cross correlation was determined over a running window $T_w = 1.1T$ with center at time-point t. In the resulting maximum peak at each t, all mitochondria in that cluster were identified and their mean TMRE signal in T_w was cross-correlated with that of each mitochondrion in the myocyte that did not belong to the cluster. Again, if the correlation coefficient was 95% or higher, the individual mitochondria were incorporated into the cluster, thus yielding the major cluster of mitochondria that are highly correlated at time t. The normalized dynamic area of the major cluster was taken as the quotient of the total dynamic cluster pixel count and the total myocyte pixel-count.

COHERENCE ANALYSIS

Temporal properties of major cluster mitochondria were examined by considering the average coherence of each cluster mitochondrion's TMRE signal with all of its nearest neighbors. Coherence values range between one and zero, indicating whether two signals oscillate synchronously at each frequency or not, respectively. Using a running window T_w, a fixed Fast Fourier Transform length of $2^{11}dt$, a frequency range for each myocyte of

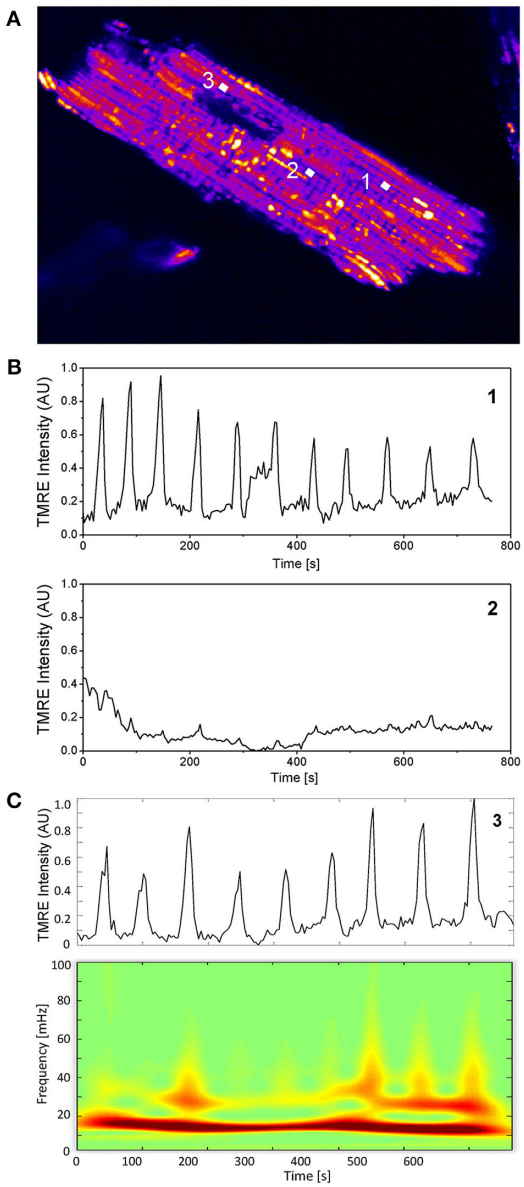

FIGURE 1 | Single mitochondrial signals and wavelet analysis. (A) Mitochondria in cardiac myocytes are densely packed and their individual TMRE signal can be extracted as previously described (Kurz et al., 2010a,b). **(B)** Two mitochondrial signals with different oscillatory patterns from different locations within the myocyte are shown. **(C)** Absolute squared wavelet transform over frequency and time of an oscillating mitochondrion. The major frequency component varies between 15 and 20 mHz.

0–100 mHz and its division into $(2^{11}/2) + 1$ segments (each segment therefore corresponding to ≈0.1 mHz), the coherence was determined between each mitochondrion and its nearest neighbors for each time point and the mean time-resolved coherence over all nearest neighbors was calculated. Consequently, the average coherence for each major mitochondrial cluster at each time point was obtained. To compare myocytes with unequal duration of recordings, the duration of oscillation of each cardiac cell was set to 1.

MITOCHONDRIAL CLUSTER AMPLITUDE

For the mean TMRE signal of the major cluster, all peaks and troughs were manually identified and the respective peak-trough amplitudes and peak-trough time differences were determined (see also Figure 4 in Kurz et al., 2010a). Normalization of peak-trough amplitudes was achieved by dividing the amplitudes through the respective maximum amplitude.

LOCAL CLUSTERING COEFFICIENT

In graph theory, clustering describes the functional topology of a network by quantifying the degree to which a set of network vertices (or constituents) resembles the graph-theoretical concept of a clique (i.e., a set of vertices in which every two vertices are connected to each other). Considering a single network vertex m, the fraction of those network vertices that are (undirectedly) connected to vertex m, with respect to the maximum number of topological neighbors of vertex m, are described by the clustering coefficient C_m. Evidently, if vertex m has m_N topological neighbors, then the maximum number of possible undirected links among these neighbors is $m_N(m_N - 1)/2$. With L_m being the number of undirected links between the neighbors of mitochondrion m, local clustering C_m is hence defined as $C_m = 2L_m/(m_N(m_N - 1))$. According to Watts and Strogatz (1998), the mean clustering coefficient of the whole complex network C is determined by the arithmetic mean of all local clustering coefficients C_m: $C = (1/M)\sum_m C_m$ with M being the number of network mitochondria.

FUNCTIONAL CONNECTIVITY

To determine the "functional" connectedness of the mitochondrial network, it is essential to determine the correlation coefficients between mitochondrial TMRE signals for each pair of mitochondria within the cardiomyocytes. For that matter, we followed a procedure by Eguiluz et al. (2005) to extract functional complex biological networks. This procedure abides by the recently introduced notion of the "spanning cluster" of oscillating mitochondria which stretches over the whole myocyte and does not necessarily involve all mitochondria (Aon et al., 2004, 2008a) as well as the concept of the dynamically changing major cluster of oscillating mitochondria described above (Kurz et al., 2010a). Two mitochondria were defined as being functionally connected at time t when the correlation coefficient of their TMRE signals in the time window T_w around t was higher than a fixed cutoff value (see also **Figure 3A** in the results section). Consequently, clustering coefficients were determined for every individual mitochondrion and, for each considered myocyte ($N = 9$), mean clustering coefficients $C(t)$ (i.e., averaged over all mitochondria at time t as in **Figure 3** or averaged over all major cluster mitochondria as in **Figure 4**) were determined for each point in time.

RANDOM NETWORK

For each myocyte, a random network was constructed that included the same number of mitochondria with the corresponding myocyte, using the Erdös–Rényi model (Erdös and Rényi, 1960). If $D_m(t)$ presents the number of links of mitochondrion m to its topological neighbors at time t (thus representing the dynamic degree of m), then each pair of

mitochondria is connected with a dynamic probability $p(t) = \sum_m D_m(t)/(M(M-1))$. To allow comparison with the real mitochondrial network, the total number of connections of the random network at each time point remains the same as that of the real network. In such a network, most mitochondria have approximately the same number of links close to the average number of links at time t, $\sum_m D_m(t)/M$ (Barabasi and Oltvai, 2004).

STATISTICS

Wavelet analysis, correlation analysis, and fitting routines were performed using Matlab v7.6.0.324 (R2008a). Further statistics were performed using OriginPro 8 SR0 v8.0724 (B724).

RESULTS

INTER-MITOCHONDRIAL "MEAN FIELD" CORRELATION

Mean-field coupling in complex non-linear biological networks of weakly coupled oscillators, such as a network of mitochondrial oscillators (Aon et al., 2006) or in other systems of coupled chemical or physical oscillators, assumes a global, all-to-all-coupling with a respective mean coupling constant (Kiss et al., 2002; Rougemont and Naef, 2006). In analogy to such a "mean field" approach, inter-mitochondrial correlation properties were examined at time t through signal correlations $c_i^{(m)}(t)$ of individual mitochondrion m with each of its N_m topological network neighbors m_i (**Figure 2**). The average correlation coefficient $\sum_i c_i^{(m)}(t)/N_m$ for each mitochondrion m was then again averaged over all network constituents to determine the overall dynamic inter-mitochondrial mean correlation $c(t)$ as $c(t) = \sum_m \sum_i c_i^{(m)}(t)/N_m$.

For each myocyte, correlation maps were created to visualize the distribution of average correlation coefficients $c_i^{(m)}(t)$ within the myocyte at time t. To achieve this, values of $c_i^{(m)}(t)$ were placed at the pixel-positions of the respective mitochondrion and missing inter-mitochondrial pixels were interpolated using the "griddata" function in Matlab v7.6.0.324 (R2008a). In **Figure 2**, only the time points with maximum and minimum values of $c(t)$ for each respective myocyte are represented. It can be seen that, for some myocytes with maximum averaged correlation over all mitochondria, individual mitochondrial correlation values close to 80% can encompass almost the entire myocyte whereas the same can be valid for values of the correlation coefficient close to 0 (e.g., mitochondrion (d) and (g) in **Figure 2**). Averaged over all myocytes ($N = 9$) and time points, the mean correlation coefficient was found to be 0.43 ± 0.07.

MITOCHONDRIAL NETWORK CLUSTERING

The mean functional inter-mitochondrial clustering coefficient was determined for different correlation cutoffs (see **Figure 3A**). Naturally, high correlation coefficient cutoffs only involve few functionally connected mitochondria whereas lower cutoffs involve a higher number of mitochondria that are thought of as being connected. Therefore, the mean clustering coefficient increases for lower cutoffs. The ensuing fitted curve f (red line in **Figure 3A**, based on equation: $f(t) = a \cdot \mathrm{Exp}[-t/b] + c$) approximately crosses the 50% value of the mean clustering

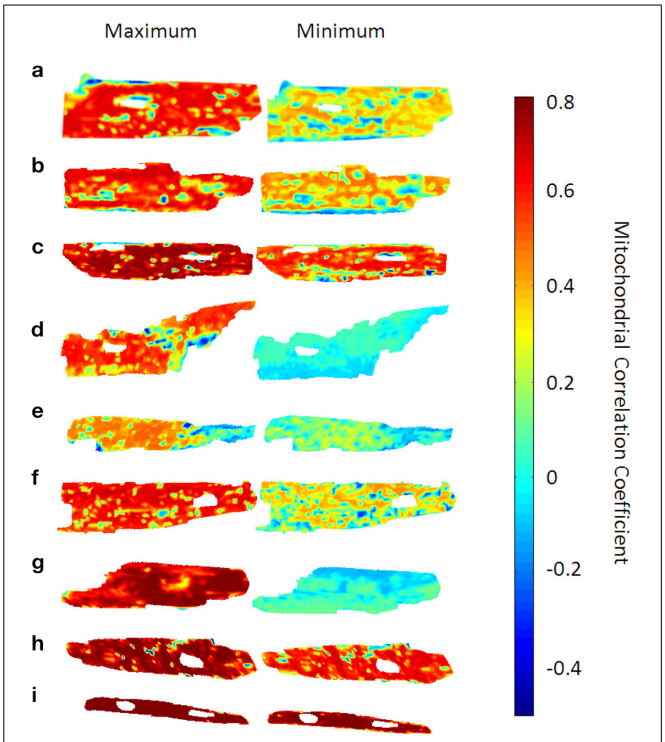

FIGURE 2 | Time-dependent mitochondrial "mean field" correlation maps for 9 cardiac myocytes. The left hand side shows the correlation maps at the time point with the highest network mean correlation $c(t)$ (see main text), whereas the right hand side shows those maps at the time point with the lowest mean correlation. Mitochondrial mean correlation values range between −0.4 and 0.8, the time-averaged mean correlation coefficient was found to be 0.43 ± 0.07. Also, some myocytes [myocytes (d) and (g)] show a prominent difference in overall correlation, while myocyte (i) does not show too pronounced correlation variability.

coefficients at correlation coefficients of 90% which also approximately corresponds to the median derivative value of the fitted curve. The fitting curve function parameters were determined as $a = -7.81 \cdot 10^{-6} \pm 1.33 \cdot 10^{-6}$, $b = -8.64 \cdot 10^{-2} \pm 0.13 \cdot 10^{-2}$ and $c = 77.65 \cdot 10^{-2} \pm 0.35 \cdot 10^{-2}$. Accordingly, and throughout the paper, two mitochondria are thought of as being connected, if their correlation exceeds 90%.

Time-normalization enables an estimate of the time evolution of the mean clustering coefficient (averaged over all mitochondria in the respective myocyte) for myocytes with unequal recording durations, and its comparison with that of random networks consisting of the same number of topological vertices and undirected links as the mitochondrial network of the respective myocyte (**Figure 3B**). Here, time-averaged mean clustering coefficients were found to be 0.500 ± 0.051 vs. 0.061 ± 0.020 for random networks, respectively.

MAJOR CLUSTER PROPERTIES IN RELATION TO MEAN MITOCHONDRIAL CLUSTERING

Individual mitochondrial signals are non-stationary in time and, therefore, wavelet transforms provide adequate means to examine the mitochondrion's signal temporal evolution

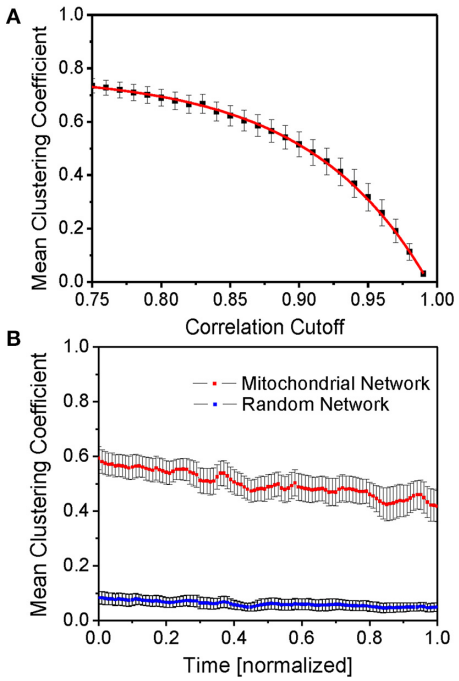

FIGURE 3 | Mean function clustering coefficient. (A) Time- and myocyte-averaged mean clustering coefficient for different cutoffs of the correlation coefficient as the defining threshold of functional connectedness. The red curve is an exponential fit curve of the form $f(t) = a \cdot \mathrm{Exp}\left[-t/b\right] + c$ (for details and fit parameters see main text). **(B)** Time evolution of the mean clustering coefficient averaged over different myocytes ($N = 9$) (red line) and for random networks on the same set of vertices and maximum degree (blue line). Evidently, the mitochondrial network, as opposed to the randomly constructed network, shows significant functional clustering.

(Grossmann et al., 1985). Following a recently described methodology (Kurz et al., 2010a), mitochondria were sorted according to the dynamic behavior of their frequencies such that clusters of mitochondria with similar frequencies could be identified. Mitochondria from the major frequency cluster were subsequently sampled and their mean temporal cluster area, amplitude and coherence determined (Kurz et al., 2010a). These spatio-temporal mitochondrial network properties are related to the functional topological network properties through the mean time-dependent clustering coefficient (see **Figure 4**).

In **Figure 4A**, it is shown that the mean clustering coefficient increases with increasing mitochondrial cluster coherence. The latter takes values between 0 and 1 at each frequency and measures the degree to which two signals are synchronous or correlated: a coherence of 1 is equivalent to perfect synchronization. It is assumed that mitochondrial clusters possess a high dynamic stability since their temporal coherence does not change significantly during the recording (Kurz et al., 2010a). The mean slope of the linear fit (red line in **Figure 4A**) was found to be 1.28 ± 0.09 with the interception point at -0.34 ± 0.06. This is not surprising since functional connectedness (as defined in the methods section) was chosen such that mitochondria are highly correlated. Higher degrees of clustering therefore correspond to a higher number of an individual mitochondrion's topological neighbors whose signals are highly correlated.

Furthermore, mitochondrial cluster frequency and normalized cluster area (see methods section) were compared against mean clustering coefficients (**Figures 4B,D**): larger cluster areas show a higher degree of functional clustering due to the fact that an increase in mitochondria with similar frequencies naturally increases the number of mitochondria that are functionally connected. The mean increase of the mean clustering coefficient versus normalized mitochondrial cluster area (in the form of the linear fit in **Figure 4D**, red line) was found as 0.30 ± 0.01, the interception point at 0.28 ± 0.01. Also, the mean clustering coefficient was considered against the mean mitochondrial cluster frequency (**Figure 4B**) and it can be seen that clusters with increasing $\Delta\Psi_m$ frequencies show lower clustering. The mean decrease of clustering versus major cluster frequency is $-10.57 \cdot 10^{-2} \pm 7.32 \cdot 10^{-2}$%/mHz (the interception point of the linear fit at 0 mHz being 0.71 ± 0.02). The result is in agreement with prior results (Kurz et al., 2010a) showing that higher cluster frequencies coincide with smaller normalized cluster area, thus, with **Figure 4D**, lower mean mitochondrial clustering coefficients (compare **Figure 4D** with Figure 2 in Kurz et al., 2010a).

Figure 4C presents the relation of topological clustering and the major cluster's amplitude, normalized to the maximum amplitude. Here, topological clustering reaches a plateau close to the value of the time-averaged mean clustering coefficient of 50% for oscillation amplitudes that are lower than 80% of the maximum amplitude of the major cluster. But, for amplitude values close to the maximum value, clustering decreases to minimal values. The linear fit in **Figure 4C** has been achieved with $f(t) = a \cdot \mathrm{Exp}\left[-t/b\right] + c$ (as in **Figure 3A**)—fit parameters were found as $a = -52.01 \cdot 10^{-8} \pm 26.97 \cdot 10^{-8}$, $b = -7.29 \cdot 10^{-2} \pm 0.28 \cdot 10^{-2}$, and $c = 51.10 \cdot 10^{-2} \pm 0.31 \cdot 10^{-2}$. This is in agreement with prior observations (Kurz et al., 2010a) where it was shown that an increase in mitochondrial cluster amplitude corresponds to lower normalized cluster area, therefore, lower mean clustering coefficients (Figure 4 in Kurz et al., 2010a).

DISCUSSION

Mitochondrial networks in cardiac myocytes show evidence of interdependence between network structure and mitochondrial metabolism (Zorzano et al., 2010), as well as properties of a complex non-linear system under pathophysiological conditions (Aon, 2010). However, the only existing quantitative analysis of the mitochondrial network's topology is based on mitochondrial reticulum models (Sukhorukov et al., 2012), whereas other models mostly considered the networks biochemical properties (Wu et al., 2007; Zhou et al., 2010). Thus, there has been no prior study of the networks functional connectedness through quantitative individual mitochondrial signal analysis.

In the present work, we have shown that the mean correlation and the mean functional clustering coefficients in mitochondrial networks are spatially and temporally variable. The latter were shown to be significantly higher than those for random mitochondrial networks with the same respective number of mitochondria and inter-mitochondrial topological connections. Furthermore, topological network properties were related to the

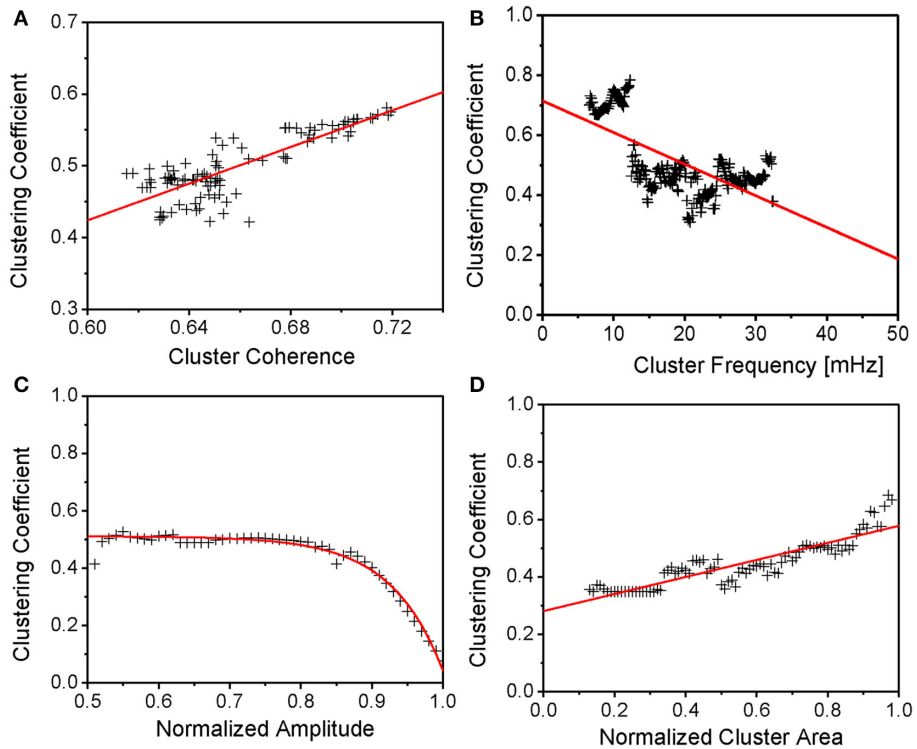

FIGURE 4 | Major Cluster Properties in Relation to Mean Mitochondrial Clustering. (A) Mean clustering coefficient vs. major cluster coherence. Though cluster coherence variability is limited, mean clustering still increases naturally for increasing cluster coherence. **(B,D)** Mean clustering coefficient vs. major cluster frequency and normalized cluster area. Clustering is shown to increase with cluster area and, therefore, number of cluster mitochondria. In analogy to Figure 2 in Kurz et al. (2010a), clustering then decreases with increasing cluster frequencies. **(C)** Mean clustering coefficient vs. normalized cluster amplitude. Clustering reaches a plateau for normalized amplitudes below about 4/5 of the maximum amplitude, whereas clustering decreases toward zero for maximum amplitudes. The latter effect might be explained by a more prolonged and incomplete repolarization in between oscillations when the relative size of the cluster increases whereas the plateau might signify a maximum of mitochondrial clustering. However, further amplitude decrease might be due to a failure of the mitochondrial network to reenergize.

spatio-temporal properties of the major mitochondrial cluster with similar cluster coherence, frequency, normalized amplitude and cluster area. It could be shown that clustering increases with the percentage area of the cluster as well as the cluster's coherence. At first sight, this seems intuitively correct, however, the concept of clustering should not be confused with the notion of mitochondrial clusters in frequency. Nevertheless, increasing the number of mitochondria with similar frequencies, as is the case in synchronizing mitochondrial networks (Aon et al., 2006), increases the number of mitochondria in the network that are functionally linked, and, therefore, mean mitochondrial clustering increases. Also, in agreement with previous results (Kurz et al., 2010a), mean mitochondrial clustering was shown to decrease with higher frequencies supporting the notion that smaller clusters of mitochondria oscillate with a higher frequency. It has been proposed that this process can be due to temporal limitations of diffusion-mediated inter-mitochondrial coupling that starts in small nuclei of excited mitochondria (Aon et al., 2006) and progresses within the coupling medium (Kurz et al., 2010a), therefore leading to a decrease in the mean cluster frequency through an increase in depolarization time, and/or due to coupled mitochondrial oscillators adjusting to a common oscillatory mode with larger clusters taking longer to synchronize.

Additionally, it has been demonstrated that increased normalized cluster amplitudes are related to a decrease in mean mitochondrial clustering. This less intuitive result can be explained by the fact that mitochondria in the major cluster show a more prolonged as well as incomplete repolarization in between oscillations when the relative size of the cluster increases. Then again, the increase of cluster-incorporated mitochondria leads to an increase of topological clustering (cf. Aon et al., 2008b). Yet, **Figure 4C** indicates that this effect comes to a halt for amplitudes below approximately 4/5 of the maximum amplitude. In this amplitude realm, mean mitochondrial clustering remains constant at its time-averaged value for decreasing cluster amplitudes suggesting a stable maximum of topological connectedness. However, it should be noted that the effect of fluorescence intensity loss can be due to limitations of the TMRE readout that might be inadequate to track correlations in the state when redox equivalents supply is insufficient to reenergize the membrane. Under these conditions, energetic/redox impairment may eventually lead to cell death.

Mitochondrial networks consist of regularly organized lattice-like mitochondria that are seen as metabolic network hubs with multiple connections to anabolic and catabolic cellular pathways (Aon et al., 2008b; Aon and Cortassa, 2012). Their coordination

was first dynamically examined in cardiac myocytes that were subjected to metabolic stress (Romashko et al., 1998; Aon et al., 2003) where it was presumed that ROS-induced ROS release acts as a coupling mediator between mitochondria to coordinate local and global cell-wide mitochondrial network behavior (Zorov et al., 2000; Aon et al., 2004). The concept of scale-free networks and its application to the mitochondrial network's functional connectivity has been the focus of recent studies (Aon et al., 2004, 2006, 2007, 2008b; Barabasi and Oltvai, 2004), which support the idea that the mitochondrial network architecture is connected to its function (Viola et al., 2009) and that metabolic supply changes can be due to morphological changes of the mitochondrial network structure, and, specifically, the morphology of mitochondrial membranes (Dimmer and Scorrano, 2006; Mcbride et al., 2006).

The present study also supports this concept in showing that topological clustering in mitochondrial networks is significant and relates directly to the network's spatio-temporal organization. It has also been shown that a mitochondrion's localization within the mitochondrial network has an effect on its functional properties (Lesnefsky et al., 1997; Kuznetsov et al., 1998). This is in agreement with the observation of strongly variable areas of mean-field correlation in mitochondrial networks as well as time-variant mitochondrial clustering. In addition, several studies could demonstrate that some individual mitochondria show independent responses to mitochondrial network dynamics (Loew et al., 1993; Duchen et al., 1998), again emphasizing mitochondrial functional heterogeneity and the complexity of the network's functional connectedness. The principle of functional connectedness applied in this study has also been used to describe brain functional networks that were shown to exhibit significantly larger clustering coefficients than those of random networks (Eguiluz et al., 2005); they are subject to intensive clinical research (see Bullmore and Sporns, 2009 for a review). Interestingly, correlations between spontaneous changes in brain activity generate very robust functional networks on timescales of seconds to minutes, i.e., in the same range as mitochondrial network dynamics (Greicius et al., 2003; Fox et al., 2005). Continuing this comparison, neuronal phase synchronization between coupled neurons was found to be highly dependent on clustering (Percha et al., 2005) and to possess self-organized critical dynamic properties (Siri et al., 2007).

The present analysis is limited to a functional analysis of the mitochondrial network. Furthermore, the present study only accounts for spatio-temporal properties of mitochondria in the major frequency clusters but neglects smaller clusters. This can be justified since the major cluster usually comprises the majority of network mitochondria that are above a percolation threshold of approximately 60% for lattice-like organized networks (cf. Aon et al., 2004). Nevertheless, the effect of long-range connections between different clusters and, therefore, the modularity of the network, will be the subject of further studies. Methods that examine the effective connectivity of the system to evaluate the causal influence of each system element on the network's behavior (see Friston, 2011 for a review), could be used to assess the connectivity between mitochondrial clusters within the myocyte or between neighboring myocytes, and consequently to examine scaling effects of mitochondrial clustering. It should also be noted that only two-dimensional slices of the cardiac myocytes have been examined here, that consequently contained only parts of the three-dimensionally arranged mitochondria. However, we would expect lateral coordination among rows of mitochondria above and below those in the focal plane to be similar to the communication between adjacent rows in the 2D plane, since the mitochondrial network has a symmetrical quasi-square lattice organization. The main source of anisotropy is the lateral versus longitudinal orientation of the mitochondria arrangement between the myofilaments, as evidenced by preferential depolarization propagation in the mitochondrial network along the axis parallel to the cardiac myocyte myofilaments (Kurz et al., 2010a).

In summary, the results of this study indicate that cardiac mitochondria constitute a collection of coupled network components that are subordinate to dynamic changes in their non-random functional connectedness.

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Complex oscillatory redox dynamics with signaling potential at the edge between normal and pathological mitochondrial function

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The time-keeping properties bestowed by oscillatory behavior on functional rhythms represent an evolutionarily conserved trait in living systems. Mitochondrial networks function as timekeepers maximizing energetic output while tuning reactive oxygen species (ROS) within physiological levels compatible with signaling. In this work, we explore the potential for timekeeping functions dependent on mitochondrial dynamics with the validated two-compartment mitochondrial energetic-redox (ME-R) computational model, that takes into account (a) four main redox couples [NADH, NADPH, GSH, $Trx(SH)_2$], (b) scavenging systems (glutathione, thioredoxin, SOD, catalase) distributed in matrix and extra-matrix compartments, and (c) transport of ROS species between them. Herein, we describe that the ME-R model can exhibit highly complex oscillatory dynamics in energetic/redox variables and ROS species, consisting of at least five frequencies with modulated amplitudes and period according to power spectral analysis. By stability analysis we describe that the extent of steady state—as against complex oscillatory behavior—was dependent upon the abundance of Mn and Cu, Zn SODs, and their interplay with ROS production in the respiratory chain. Large parametric regions corresponding to oscillatory dynamics of increasingly complex waveforms were obtained at low Cu, Zn SOD concentration as a function of Mn SOD. This oscillatory domain was greatly reduced at higher levels of Cu, Zn SOD. Interestingly, the realm of complex oscillations was located at the edge between normal and pathological mitochondrial energetic behavior, and was characterized by oxidative stress. We conclude that complex oscillatory dynamics could represent a frequency- and amplitude-modulated H_2O_2 signaling mechanism that arises under intense oxidative stress. By modulating SOD, cells could have evolved an adaptive compromise between relative constancy and the flexibility required under stressful redox/energetic conditions.

Keywords: ROS signaling, mitochondrial energetic/redox, complex oscillations, Hopf bifurcations, physiological and pathophysiological behavior, redox environment

INTRODUCTION

Poised at the convergence of most catabolic and anabolic pathways, mitochondria are at the center of heterotrophic aerobic life, representing a hub in the cellular metabolic network (Aon et al., 2007a; Aon and Cortassa, 2012; Cortassa and Aon, 2013; Kembro et al., 2014). The energetic functions performed by mitochondria face the unavoidable redox hurdle of handling huge amounts of O_2 while keeping their own as well as the cellular redox environment. Mitochondria produce ∼85–90% of cellular reactive oxygen species (ROS) (Chance et al., 1979; Shigenaga et al., 1994; Balaban et al., 2005), while supplying the bulk of ATP demanded by the organs in the human body. The heart consumes proportionately most of the O_2 on a specific basis with respect to the whole human body (Rolfe and Brown, 1997) thereby becoming especially vulnerable to oxidative damage. Although myocardial function declining with age, as well as the ability of the heart to tolerate stress (Lakatta and Sollott, 2002), are not understood mechanistically, mitochondrial dysfunction, oxidative stress and the accumulation of oxidant-induced damage are major contributing factors (Fannin et al., 1999; Suh et al., 2003; Judge et al., 2005a,b).

Originally considered an unavoidable and dangerous byproduct of oxidative phosphorylation (OxPhos), more recently we have become aware of the crucial role played by ROS signaling in key cellular functions. If under control, H_2O_2 becomes recognized as a specific signaling molecule, but beyond physiological limits it can readily become damaging. Under pathophysiological conditions, excessive ROS levels can occur due to either alterations in production, overwhelming of antioxidant defenses, or both (Aon et al., 2003, 2007a; Jones and Go, 2010). However,

levels compatible with signaling are attained when production and scavenging of ROS are balanced within mitochondria and cells.

The redox environment (RE) determines the relationship between mitochondrial respiration and ROS. At maximal respiratory rate, mitochondrial ROS emission trends to a minimum and exhibits a clear dependence on the RE, from ∼400 to 900 mV·mM in state 4 respiration and ∼500 to 300 mV·mM in state 3 respiration (Cortassa et al., 2014). The dependence of ROS on mitochondrial respiration involves two terms: production and emission; whereas the former depends on respiration (i.e., the rate of electron transport through the respiratory chain) the latter relies on the balance between the production and scavenging roles. The ROS scavenging capacity is tightly linked to the redox-energetic status of mitochondria. NAD(P)H is the main electron donor to the antioxidant systems, but its generation depends on NADH, which exerts a dual redox and energetic role through transhydrogenase and complex I acceptors, respectively.

Recent data highlight the dominant role exerted by the glutathione (GSH) and thioredoxin (Trx) scavenging systems on H_2O_2 emission dynamics from mitochondria (Stanley et al., 2011; Kembro et al., 2013), especially under state 3 respiration when the energetic output is maximal (Aon et al., 2012; Cortassa et al., 2014). These data suggest that the GSH/Trx systems continuously scavenge ROS produced in the respiratory chain, thereby demonstrating that the antioxidant systems play a determinant task in the dynamics of H_2O_2 release by mitochondria. In this scenario, the emerging role of mitochondria as signaling organelles and ROS as signaling molecules increases the importance of understanding the dynamics of ROS emission and its role in normal as well as stress conditions. Mitochondria were shown experimentally and theoretically to be autonomous oscillators (Aon et al., 2003, 2008b; Cortassa et al., 2004; Kurz et al., 2010a; Qu, 2013) thus potentially representing a frequency- and amplitude-modulated signaling mechanism that could connect energetics to ROS-activated signaling pathways, including those responsible for regulating gene transcription (Morel and Barouki, 1999; Misra et al., 2003; Aon et al., 2006, 2007a, 2008a).

Duplication of antioxidant defense systems in multiple compartments can be an efficient salvage mechanism in response to oxidative bursts, and as a modulator of ROS dynamics. Superoxide dismutase (SOD) represents a relevant example of duplicated ROS scavenging systems localized in distinct compartments. Mammals have three isoforms of SOD present in the extracellular Cu, Zn SOD (SOD3), cytoplasmic Cu, Zn SOD (SOD1) and the mitochondrial Mn SOD (SOD2), compartments. Together they constitute the major antioxidant defense systems in charge of safely modulating O_2^-. Exposure to oxidants can act as a signal to increase the activities and expression of antioxidant enzymes (Rodriguez et al., 2004), and as a result an increase in antioxidant enzyme activity with age is expected to help protect tissues from oxidative stress (Judge et al., 2005a).

Compartmentalization is relevant in the control of ROS levels and the redox environment (Kembro et al., 2013), but its role in the dynamics of mitochondrial signaling is unknown. Although each subcellular compartment exhibits its own dynamics, the interdependence of their permeant redox status is mediated by the exchange of redox species (e.g., GSH, ROS). A previous version of our computational model of mitochondrial function showed frequency- and amplitude-modulated oscillations (Cortassa et al., 2004). These autonomous oscillations could span several orders of magnitude (milliseconds to several hours) by simply changing one parameter, e.g., the SOD concentration in the extra-mitochondrial compartment (Cortassa et al., 2004; Aon et al., 2006, 2008b). However, unexplained in this early model formulation was the impact exerted by the duplication of SODs in mitochondrial matrix and cytoplasm, and the exchange rates of O_2^-, H_2O_2, and GSH between compartments. Consequently, in the present work we investigate the role played by the compartmentalization of SODs on the oscillatory dynamics of H_2O_2. We focus on SOD1 and SOD2 because of their demonstrated critical role in cell physiology, as well as whole organism survival, lifespan, and disease states (Antila and Westermarck, 1989; Tribble et al., 1997; Sun and Tower, 1999; Craven et al., 2001a,b; Melov et al., 2001; DeRubertis et al., 2004; Sun et al., 2004; Kowluru et al., 2006a,b; Lu et al., 2009; Massaad et al., 2009a,b; Usui et al., 2009, 2011; Fukai and Ushio-Fukai, 2011).

MATERIALS AND METHODS

COMPUTATIONAL MODEL

A two-compartment mitochondrial energetic-redox (ME-R) model (Kembro et al., 2013) was utilized to assess the influence of ROS production and antioxidant systems on the period, amplitude and waveform of mitochondrial oscillations. The ME-R model incorporates four main redox couples [NADH/NAD^+, NADPH/$NADP^+$, GSH/GSSG, Trx(SH)2/TrxSS]. Superoxide dismutases (SOD) and other scavenging systems—glutathione, thioredoxin, catalase—distributed in mitochondrial matrix and extra-matrix compartments, and transport between compartments of ROS species (superoxide: O_2^-, hydrogen peroxide: H_2O_2), and GSH are also taken into account.

The model also accounts for respiratory flux from substrates of complex I and complex II, pH effects on equilibrium constants and enzyme activity, ion dynamics (Wei et al., 2011), the shunt of electrons from the respiratory chain toward the generation of O_2^- (Shunt), and a ROS-activated anion efflux pathway across the inner membrane (Cortassa et al., 2004). Synthesis of NADPH from $NADP^+$ and NADH via isocitrate dehydrogenase 2 (IDH_2) and transhydrogenase (THD), respectively, are also included in the ME-R model.

The scheme for the integrated model is shown in **Figure 1**, and its complete description as well as parameterization is described elsewhere (Kembro et al., 2013).

MODEL SIMULATIONS

All studies were performed using the parametric setting with which the ME-R model was able to simulate different experimental situations (Kembro et al., 2013), with the exception of the concentrations of Mn SOD and Cu, Zn SOD, and Shunt values.

Numerical integration of model equations (ODE15s) was performed with MatCont 2.4 (Dhooge et al., 2008) in MATLAB 7.1, until steady-state solutions were obtained (i.e., when the magnitude of each time derivative was $<10^{-10}$). Steady-state values

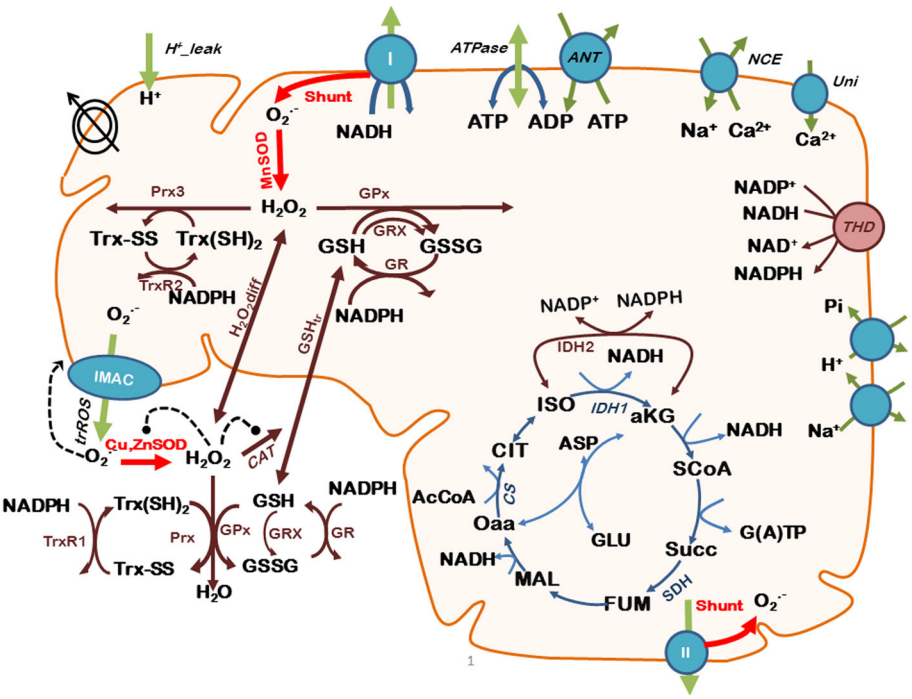

FIGURE 1 | Scheme of the two-compartment ME-R model accounting for mitochondrial energetic and redox processes, their interactions, and transport between compartments. The model takes into account oxidative phosphorylation (OxPhos) and matrix-based processes in mitochondria as well as in the extra-mitochondrial compartment. In addition to energy metabolism and ion transport (H^+, Ca^{2+}, Na^+, Pi), the model accounts for O_2^- being produced in the mitochondrial electron transport chain from both complex I- and complex II-derived electron transport. O_2^- may be dismutated to H_2O_2 by matrix-localized superoxide dismutase (MnSOD) or be transported to the extra-mitochondrial compartment through the inner membrane anion channel (IMAC), where it will be scavenged by Cu,ZnSOD. H_2O_2 can either diffuse from the matrix or be scavenged by the large capacity glutathione (GSH) and thioredoxin (Trx) systems, or by catalase (CAT) in the extra-mitochondrial compartment. Glutaredoxin (Grx) accounts for the recovery of glutathionylated proteins in the matrix. *Key to symbols*: Concentric circles with an arrow across represent the $\Delta\Psi_m$. Dotted arrows indicate regulatory interactions either positive (*arrowhead*) or negative (●--). "Shunt" indicates the fraction of electrons from respiration diverging toward O_2^-. The red arrows highlight the model state variables (SODs and Shunt) that will be evaluated with respect to their impact on mitochondrial oscillations. Modified from Kembro et al. (2013).

of each state variable were then used as input for performing bifurcation and continuation analysis performed with MatCont 2.4 (Dhooge et al., 2008) in MATLAB 7.1. This software is used to determine the dependence of steady-state solution properties (type and stability) on model parameters. Eigenvalues characterizing the bifurcation properties of the ME-R model were also analyzed with MatCont 2.4. For stability analysis, the Shunt was utilized as the bifurcation parameter at fixed concentrations of mitochondrial superoxide dismutase (Mn SOD) and extra-mitochondrial superoxide dismutase (Cu,Zn SOD).

Time series analysis was performed on series with a duration of $1.6.\ 10^7$ ms obtained by numerical integration of model equations using absolute tolerance of 10^{-14} and relative tolerance of 10^{-9}. The solutions were then evaluated according to Kierzenka and Shampine (2011) in MATLAB R2013a to obtain time series with constant sampling frequency at 1 ms. The system was simulated for an extended period of time (i.e., at least $2.\ 10^9$ ms) to ascertain the achievement of stationary time series. These time series were then analyzed by power spectral analysis using the Fast Fourier Transform (FFT) subroutine of Matlab. Due to the stationarity of the time series they were not preprocessed or filtered.

RESULTS

EXTRA-MITOCHONDRIAL CuZnSOD DETERMINES OSCILLATORY MITOCHONDRIAL DYNAMICS AT THE EDGE BETWEEN FUNCTIONAL AND PATHOLOGICAL BEHAVIOR

We investigated the dependence of the mitochondrial dynamic behavior (onset and extent of oscillatory behavior) as a function of three key model parameters (concentrations of Mn SOD and Cu, Zn SOD, and Shunt). Mitochondrial dynamics evolves toward a steady state (i.e., fixed point attractors) or oscillations (i.e., limit cycles) depending on the antioxidant capacity of the mitochondrial and extra-mitochondrial compartment via Mn SOD and Cu, Zn SOD, respectively, when Shunt (i.e., ROS production) is increased. We analyzed the appearance of three distinct mitochondrial states: (1) functional (i.e., highly reduced NADH, polarized membrane potential and minimum ROS release), (2) pathological (i.e., highly oxidized NADH, depolarized membrane potential and high ROS release), and (3) oscillatory (i.e., oscillations in main bioenergetic variables such as ROS release, membrane potential, tricarboxylic acid (TCA) cycle intermediates, and antioxidant systems).

Figure 2 depicts a more detailed exploration of mitochondrial redox (NADH) behavior as a function of SODs and Shunt using stability analysis. An extensive oscillatory region delimiting functional from pathological domains of mitochondrial behavior appears as a function of increasing ROS production, i.e., higher Shunt (**Figure 2B**). This oscillatory region becomes more confined as the antioxidant capacity of Cu, Zn SOD in the extra-mitochondrial compartment is enhanced (**Figures 2D,F**).

The bifurcation diagrams evolve from smoother to steeper S-shapes depending on the concentration of Cu, Zn SOD (**Figures 2A,C,E**). Unlike the typical S-shape behavior exhibited by bistable systems, the transition between the upper (reduced) and lower (oxidized) branches of NADH states in the two-compartment ME-R model is not done abruptly at limit points (Aon and Cortassa, 1997; Cortassa et al., 2004). In contrast, the thin line connecting upper and lower branches of steady states in the bifurcation diagrams from **Figure 2** exhibits both an unstable focus and a stable limit cycle (see insets i–iii from **Figure 2**). According to the stability analysis, the limit cycles appear after Hopf bifurcations (HBs) exhibiting 2 and up to 4 positive eigenvalues corresponding to the real component of the complex imaginary numbers characterizing HBs, i.e., the higher the Cu, Zn SOD concentration the higher the number of positive eigenvalues (**Figures 2B,D,F**). A positive eigenvalue implies sustained oscillations whereas a higher number of them suggest different types of oscillatory behavior (see **Figure 5** below).

Combinations of higher Mn SOD and/or Cu, Zn SOD concentrations bestow a higher tolerance to ROS produced before the system transitions toward oscillations or steady (but depolarized) states (**Figure 2**). Low values in either class of SOD can be reciprocally compensated by higher values of the other thus preserving conditions compatible with life under oxidative stress (**Figures 2B,D,F**). Consequently, it appears that both SODs can compensate each other to maintain functionally compatible dynamic behavior. Qualitatively, the dynamic behavior of the model agrees with experimental evidence showing that either increasing the concentration of ROS scavengers, or inhibiting respiration to decrease mitochondrial ROS production, inhibits oscillations in $\Delta\Psi_m$ by stabilizing the polarized steady state, or by distancing the mitochondrial network from criticality, i.e., preventing ROS accumulation to the critical threshold (Aon et al., 2003, 2004; Cortassa et al., 2004).

COMPLEX OSCILLATORY BEHAVIOR AT THE EDGE OF NORMAL AND PATHOLOGICAL MITOCHONDRIAL BEHAVIOR

To better characterize mitochondrial oscillations at the edge region, we analyzed frequency (1/period) and amplitude as a function of different parametric combinations of SODs and Shunt. Within the oscillatory domain, an increase in the concentration of Cu, Zn SOD or Mn SOD (**Figure 3A**, compare green and black lines) or a decrease in Shunt (**Figure 3A** compare green and blue lines) results in lower frequency oscillations. Interestingly, different combinations of these three parameters can lead to oscillations with the same frequency (**Figure 3A**, dotted line), although not necessarily with the same amplitude (**Figures 3B, 4**). For example, model simulations can reproduce the frequency of experimentally observed oscillations (∼0.01 Hz, equivalent to a period of ∼100 s) (Cortassa et al., 2004) for at least four distinct parametric combinations (**Figure 3**).

Considering the oscillations obtained under the conditions specified in **Figure 3A**, we examined the dependence of their amplitude vs. frequency. A double-log plot revealed an inverse relationship of amplitude vs. frequency (from >0.01 Hz) in oscillations of energetic ($\Delta\Psi_m$, succinate) (**Figures 4A,B**) and redox (O_2^-, H_2O_2) (**Figures 4C,D**) variables obtained at different Cu, Zn SOD concentrations. According to this inverse relationship, an increase in the frequency (corresponding to a decrease in CuZnSOD concentration shown in **Figure 3A**) results in a decrease in the amplitude of the oscillations.

Under oxidative stress (Shunt = 4%), increasing Mn SOD at low Cu, Zn SOD results not only in changes in frequency and amplitude, but also in the complexity of the oscillatory waveform (**Figure 5**). The shape of the oscillations in H_2O_{2i} concentration shifted from a spike- to a sinusoidal-like wave form (**Figures 5A,B**). When the oscillatory signal corresponding to 10.2 μM MnSOD was analyzed by power spectral analysis, a high sharp peak in the frequency domain was observed at ∼0.035 Hz, followed by harmonics of slightly lower values (**Figure 5C**). Mathematically, this time series shows similarities with a Dirac comb (also called spike train) (Kanasewich, 1981) that reflects the appearance of sharp spikes at 29 s intervals in H_2O_{2i} concentration (**Figure 5A**).

At 17 μM MnSOD, three low-frequency components (∼0.0055, 0.011, and 0.0165 Hz) of decreasing power can be observed in the frequency domain (**Figure 5C**); the ∼0.0055 Hz frequency corresponds to the predominant waveform with a period of 182s (**Figure 5A**). Other harmonic frequencies may contribute to the complexity of the waveform (**Figure 5C**, inset).

At ∼21.7μM MnSOD, a first lower spike at 0.00225 Hz is followed by two major spectral components of lower (∼0.00444 Hz) and higher (∼0.00894 Hz) frequencies (**Figure 5C**), equivalent to periods of ∼444, 225, and 112 s, respectively. These first two spectral components clearly reflect the period doubling process, while the period of 112s marks the appearance of smaller intermediate peaks (**Figure 5A**). Overall, per cycle of 444s, two large and two small peaks are observed corresponding to H_2O_{2i} concentration values of 0.535, 0.068, 0.729, and 0.077 μM. The complexity of the waveform is further underscored by a large number of contributing harmonic frequencies of different magnitudes (**Figure 5C**, inset).

Oscillations in $\Delta\Psi_m$(**Figure 5D**) and succinate (**Figure 5E**) also show progressively complex waveforms for increasing concentrations of MnSOD (**Figure 5D**). However, the waveform complexity of $\Delta\Psi_m$ (**Figure 5D**) is lower than in H_2O_{2i} (**Figure 5A**) and succinate (**Figure 5E**).

Phase space 3D projections of the state variables H_2O_{2i}, $\Delta\Psi_m$ and succinate demonstrate their complex dynamic interrelationships. This can be judged by the shape of the attractors (**Figure 6**) that exhibit the highest intricacy at the maximal concentration of MnSOD tested (**Figure 6C**; see also the corresponding time series in **Figures 5A,D,E**).

Overall, the results obtained indicate that the complexity of the oscillations waveform is enhanced as a function of increasing oxidative stress conditions.

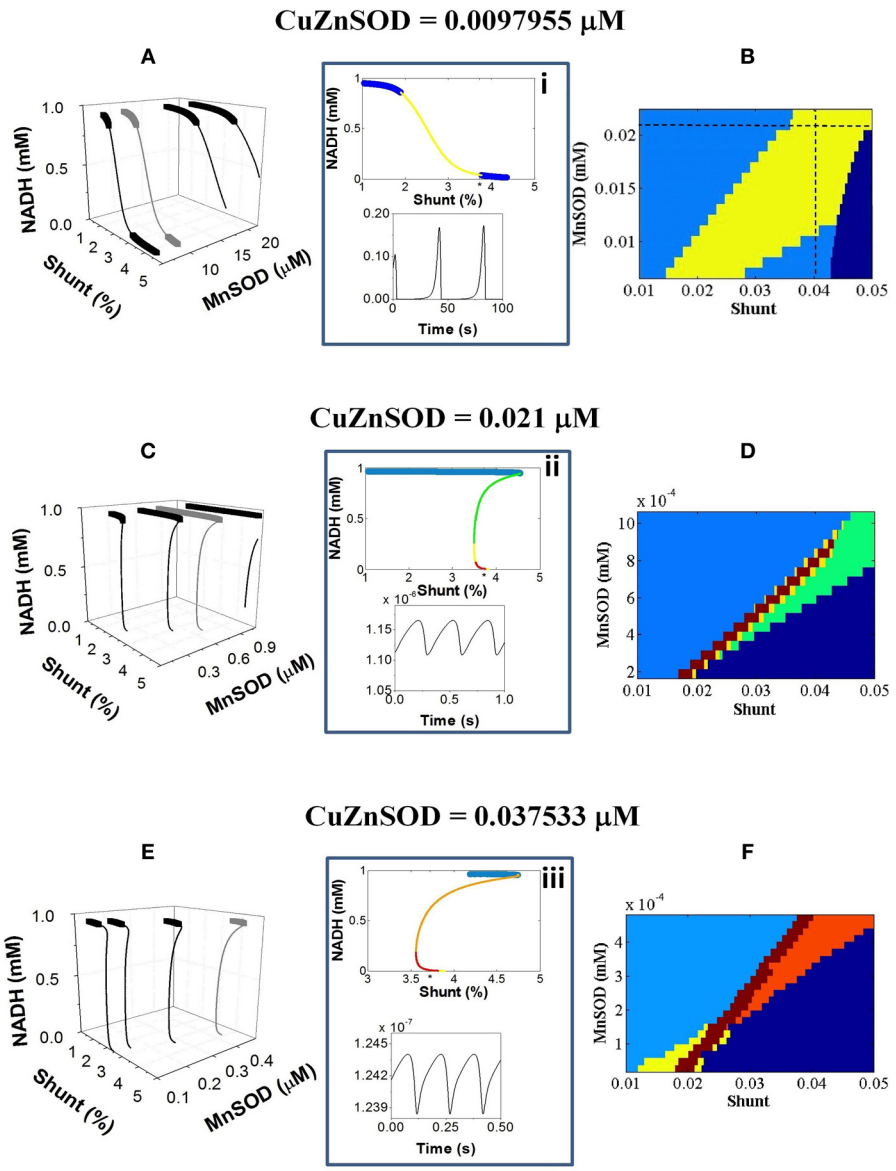

FIGURE 2 | NADH dynamic behavior as a function of mitochondrial ROS production and scavenging, at different antioxidant capacity of the extra-mitochondrial compartment. The model behavior was analyzed by stability analysis as described in Methods. **(A,C,E)** Depicted are the bifurcation diagrams showing an upper branch of steady states in which NADH is predominantly reduced, and a lower branch in which NADH is mainly oxidized. Thick lines correspond to stable steady-state behavior whereas thin lines denote stable limit cycle (oscillatory) behavior and an unstable focus. Hopf bifurcations mark the transition from steady to oscillatory dynamics. Further analysis of the eigenvalues obtained in the stability analysis enabled a detailed description of the oscillatory region. Insets i–iii depict an example of eigenvalues for the bifurcation diagrams shown in gray in the panels **(A,C,E)** at the left. Information of the maximum eigenvalue observed for a given parametric combination is represented in the colored plots in the **(B,D,F)** panels at the right. In the stable region of the diagram denoted in light blue (normal behavior), all negative real values (i.e., stable steady states corresponding to fixed point attractors) were found whereas in the oscillatory region at least one positive real eigenvalue could be determined (i.e., sustained oscillations corresponding to a stable limit cycle). Green, yellow, orange and red colors code for 1, 2, 3, or 4 real positive eigenvalues, respectively. Dark blue indicates the pathological domain corresponding to non-functional ("dead") mitochondria. The asterisk (*) on the *x-axis* of top panel from the middle insets (i–iii) corresponds to the Shunt value at which the time series represented at the bottom panel was obtained. In panels **(B,D,F)** the maximum number of positive eigenvalues found for each parametric combination is represented with the same color code used in the insets. The dotted lines in panel **(B)** correspond to the parametric combinations giving rise to complex oscillations shown in **Figure 5**.

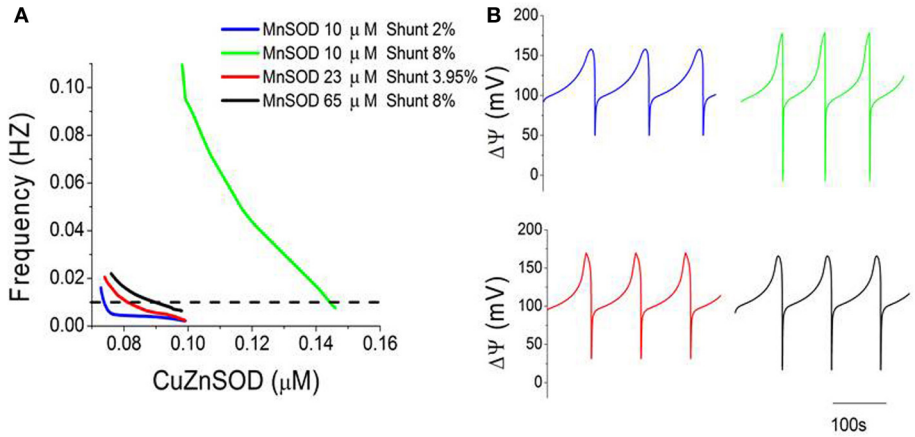

FIGURE 3 | Three-way modulation of the oscillations' frequency in mitochondrial membrane potential. (A) The frequency (1/period) of mitochondrial oscillations as a function of increasing concentrations of CuZnSOD at four different combinations of MnSOD and Shunt. Notice that the oscillator may attain the same frequency (0.01 Hz, or 100 s period) with different combinations of the three parameters (MnSOD, CuZnSOD, and shunt) as indicated by the dotted line. **(B)** Displayed are the time series corresponding to the four parametric combinations shown in **(A)** at a frequency of 0.01 Hz.

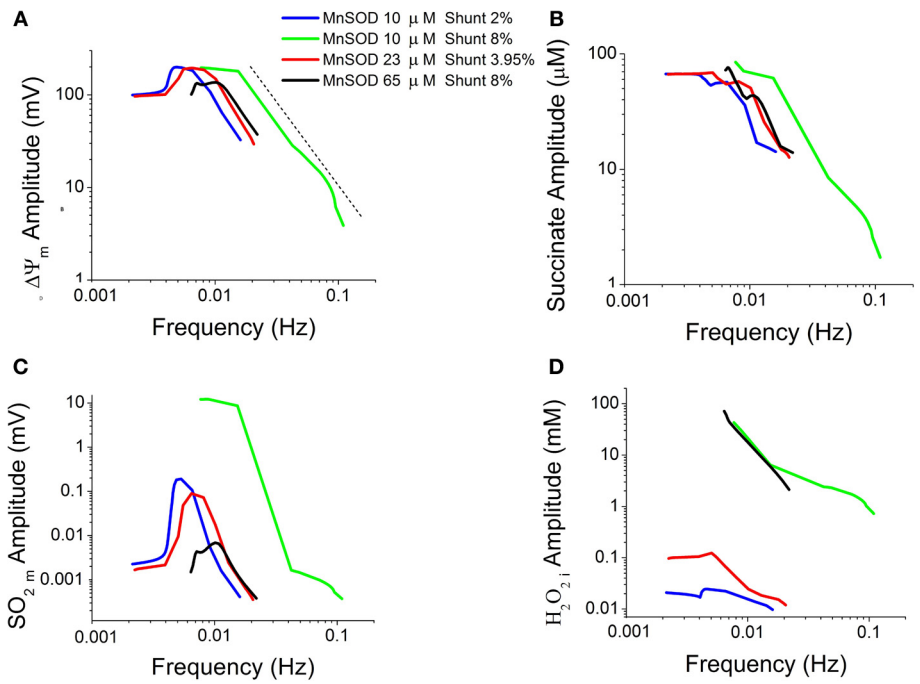

FIGURE 4 | Double-log plot of oscillation amplitude vs. frequency for different time series. Characterization of oscillatory behavior in energetic (mitochondrial membrane potential, $\Delta\Psi_m$, succinate, Succ) and redox (mitochondrial superoxide, SO_2m, and extra-mitochondrial hydrogen peroxide, H_2O_{2i}) variables obtained at increasing concentrations of CuZnSOD as described in **Figure 3A**. Depicted are the logarithm of the oscillations amplitude as a function of their frequency in $\Delta\Psi_m$ **(A)**, Succ **(B)**, SO_2m **(C)**, and H_2O_{2i} **(D)**. The dotted line in panel **(A)** indicates the linear trend in the double-log plot.

DISCUSSION

The main contribution of the present work is to show that the interplay of Cu, Zn SOD (SOD1) and Mn SOD (SOD2) activities determines the appearance of complex oscillations in mitochondrial dynamics. The complexity of the oscillations is characterized by at least more than one period, amplitude and/or type of waveform (e.g., spikes, sinusoid) and increased at high ROS production while the antioxidant capacity of the periplasmic-cytoplasmic compartments remained low. Under these conditions, the combination of SOD activities in both

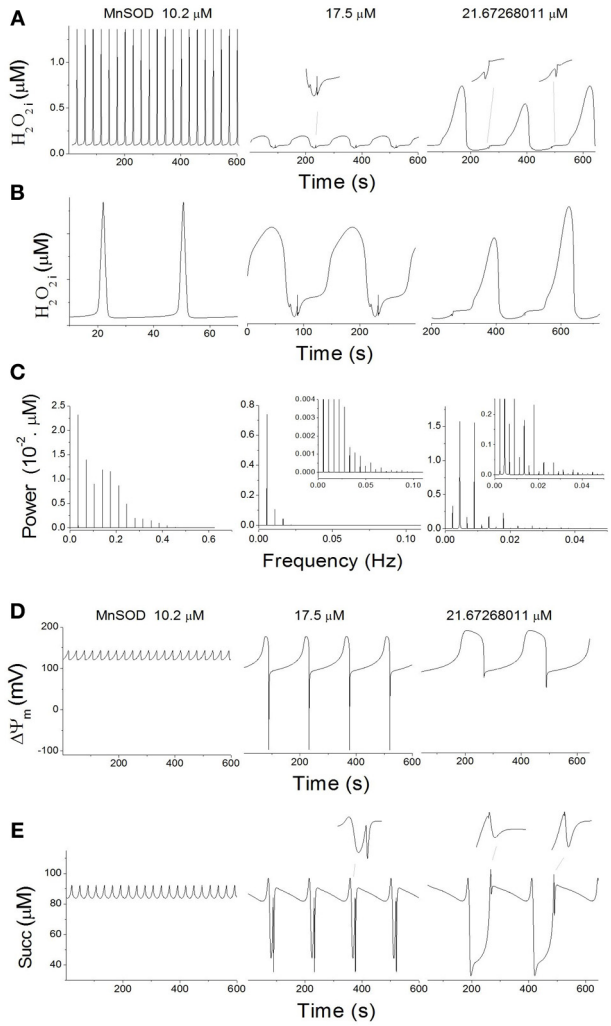

FIGURE 5 | Complex mitochondrial oscillatory dynamics. Oscillatory dynamics in H_2O_{2i} **(A)**, $\Delta\Psi_m$ **(D)**, and Succ **(E)** at increasing concentrations of MnSOD at constant 9.7 μM CuZnSOD and 4% Shunt (see dotted lines in **Figure 3A**). **(B)** Depicted are the time series from panel **(A)** after magnification and rescaling to highlight the increase in complexity of the oscillatory waveforms. **(C)** Power spectral analysis of the time series from panel **(A)**. **(D)** Power spectral analysis of the time series from panel **(A)**, performed with a time series of 1.6×10^4 s at a constant sampling interval of 1 ms.

compartments defines an "edge" region that delimits normal from pathological mitochondrial states. Complex oscillations occur within the "edge" region, presenting a distinct number of amplitudes and frequencies that appear inversely related when represented in a double log plot (**Figure 4**).

Of note is that none of the other parameters from our model, apart from the three studied herein, were capable of eliciting oscillatory behavior. The range of parametric variation in "Shunt" and SOD concentrations utilized in the present work are within realistic ranges. "Shunt" was varied between 0.1 and 8% and the extent of electron diversion from the respiratory chain to produce ROS reportedly ranged from 0.15 to 11% of the O_2 consumption flux (Boveris et al., 1972; Chance et al., 1979; St-Pierre et al., 2002; Hoffman and Brookes, 2009; Aon et al., 2012), depending on species and whether mitochondria are in respiratory states 4 [zero ADP] or 3 [ADP present] (Aon et al., 2012). As for the SOD concentrations, values reported are ∼0.5 μM (McAdam et al., 1977; Chance et al., 1979; Hsu et al., 1996) and we used a range of concentrations between 0.009 and 0.16 μM for Cu, Zn SOD, and 0.1 and 65 μM for Mn SOD.

In the model, it is noteworthy that the transition between steady state and oscillatory dynamics is shown to occur in a parametric domain of ROS production and scavenging compatible with values found in nature. The ME-R model with antioxidant arrays in both compartments renders O_2^-, and H_2O_2 levels in the pM to nM range (Kembro et al., 2013). Thus, the oscillatory release of H_2O_2 from the mitochondrial compartment in the ME-R model possesses modulatory potential in both amplitude and frequency that, under critical oxidant stress, may function as a signal for redox-modulated processes (Aon, 2013; Cortassa and Aon, 2013).

A relevant example of redox signaling is represented by the regulation of protein activity and the transduction of signals to downstream proteins through oxidative modification of reactive cysteine residues by ROS, and more specifically H_2O_2 (Finkel, 2000; Paulsen and Carroll, 2010; Aon, 2013; Kembro et al., 2014). A recent example of redox signaling involving H_2O_2 was shown in the synchronization of thousands of bacterial colonies (Prindle et al., 2012). There, two synergistic modes of communication appear to be involved: quorum sensing (correlated to population density within a colony) that can produce N-acyl homoserine lactones as signaling molecules, and redox signaling (H_2O_2 vapor) between colonies (Prindle et al., 2012). The stronger, yet short-range, quorum sensing appears to be necessary to coherently synchronize the weaker, yet long-range, redox signaling. Local and long-range effects of signaling mechanisms, across organelles within cells and cells within populations have also been shown in cardiac and yeast cells (Aon et al., 2007b, 2008a; Lloyd and Murray, 2007; Roussel and Lloyd, 2007). These are yet other examples showing that the mechanism of functional synchronization across temporal and length scales are universal among organisms separated by billions of years of evolution (Lloyd et al., 2012).

The emergence of complex oscillatory behavior within the "edge" region, a major finding of this study, is determined by the interplay between the antioxidant powers granted by SOD1 in the extra-mitochondrial compartment and the balance of ROS production and scavenging within mitochondria (i.e., Shunt and MnSOD, respectively). Given the exchange of ROS species between compartments, the compartmentalization of SODs and their relative activities play a significant role in defining the extent of functional vs. pathological behavior, as well as the appearance of the "edge" region between both, populated by oscillatory dynamics. This main result is shown schematically in **Figure 7**. Oscillations occurred in a restricted region of the parametric space defined by the SODs and ROS production in the respiratory chain (denoted with light brown in **Figure 7**, which is a scheme of the results displayed in **Figure 2**). The oscillatory domain locates at the edge between normal and pathological states of mitochondria, as a function of the two

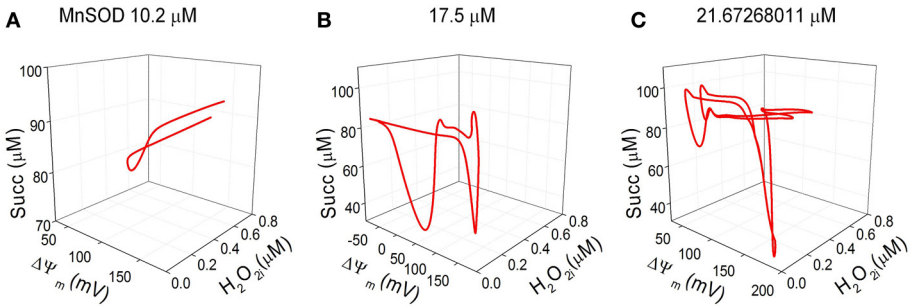

FIGURE 6 | Three-dimensional (3D) phase space projections of energetic and redox state variables. 3D phase space plots depicting the interrelationship between the dynamic trajectories of the state variables H_2O_{2i}, $\Delta\Psi_m$ and succinate for MnSOD **(A)** 10.2, **(B)**17, and **(C)** ~21.7 μM. The attractors described in the 3D phase plots correspond to the same time series shown **Figures 5A,D,E**.

parameters from the mitochondrial compartment: MnSOD vs. ROS production (shunt). Interestingly, the oscillatory domain moves toward the bottom of the plot in **Figure 7**, when CuZnSOD from the extra-mitochondrial compartment increases (**Figure 7**, inset). This result suggests that the higher the antioxidant capacity of the periplasm-cytoplasm, the larger the parametric space compatible with functional behavior. In addition, the oscillatory domain defining the edge between normal vs. pathological is also displaced toward more restricted parametric combinations. As a result when Cu, Zn SOD concentration increases, the ability of the two compartments to tolerate higher mitochondrial ROS production is enhanced, even at low concentrations of MnSOD.

Under functional conditions, mitochondria exhibit stable steady states (**Figures 2B,D,F**, light blue color) that may extend to the "edge" behavioral regimen (**Figures 3B,D,F**, various colors). The extent and the transition to the edge are determined by the extra-mitochondrial SOD1 activity, and its interplay with SOD2 and ROS production from the mitochondrial compartment (**Figures 2**, **7**). The functionally compatible "edge" domain exhibits conspicuous behavior. On the one hand, although the overall dynamic landscape is S-shaped it does not belong to classical bistable systems since abrupt transitions do not occur between stable and unstable states (**Figure 2**). Instead, a more or less gradual transition between branches of stable steady states and oscillatory ones happen. On the other hand, inside the "edge" region, the model dynamics exhibits a rich variety of bifurcation properties as revealed by the existence of several Hopf bifurcations (i.e., a signature of limit cycle, oscillatory behavior) with manifold positive eigenvalues (**Figure 2**).

The amplitude and frequency components of the oscillations obtained at different Cu, Zn SOD concentrations are inversely related when represented in a double log graph (**Figure 4**). This behavior is critically dependent on SOD activities through their impact on the balance between ROS production and ROS scavenging. This is exemplified in **Figure 4**, where the relationship between the rate of mitochondrial superoxide, SO_2m, production and its dismutation by MnSOD appear to be the difference responsible for the "kinks" depicted in **Figure 4C**. In particular, this change in behavior of the amplitude vs. frequency relationship in the SO_2m oscillation is given by the drastic difference that occurs at high rates of ROS production (8% Shunt)

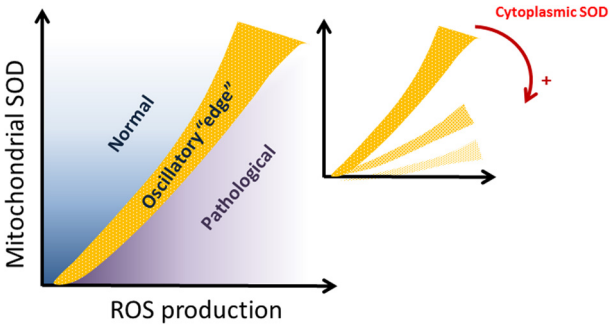

FIGURE 7 | Schematic representation of the dependence of mitochondrial dynamics on SODs compartmentalization. The domain delimiting normal from pathological mitochondrial function (represented in light brown) corresponds to the "edge" oscillatory region. The "edge" domain is characterized by the existence of complex oscillations. The inset shows how the "edge" is displaced toward the bottom of the graph as the antioxidant capacity given by CuZn SOD in the periplasmic-cytoplasmic (extra-mitochondrial) compartment increases. Red arrow and plus sign, indicate increasing concentrations of cytoplasmic SOD.

between the green and black traces in **Figure 4C**, where the former corresponds to lower MnSOD (10 μM) than in the latter (65 μM) (**Figure 4**). In low MnSOD, the SO_2m oscillations amplitude first rises to then decrease as frequency increases (elicited by decreasing CuZnSOD concentrations; **Figure 3**). In principle, this deviation from a straightforward inverse relationship can be explained by a dynamic mismatch between the rates of SO_2m production and dismutation coupled to the dependence of ROS transport between compartments on the concentration gradient of these molecules across the membrane, as accounted for by the model (Kembro et al., 2013).

The inverse amplitude vs. frequency relationship was demonstrated previously (Aon et al., 2006) and is confirmed by the present, more elaborate, ME-R model (Kembro et al., 2013). The likelihood of high-frequency, low amplitude oscillations in mitochondrial ROS and $\Delta\Psi_m$ was predicted from a computational model of the mitochondrial oscillator (Cortassa et al., 2004) and later experimentally demonstrated in cardiomyocytes (Aon et al., 2006) and oscillating, self-synchronized, yeast

cultures (Murray and Lloyd, 2007; Roussel and Lloyd, 2007; Aon et al., 2008a). Theoretical simulations indicated that the mitochondrial oscillator's period can be modulated over a wide range of time scales (Cortassa et al., 2004; Aon et al., 2006, 2008b). Although the frequency distribution is broad under normal conditions, the long-term temporal correlations of the mitochondrial network could theoretically allow a change in one time scale to be felt across the frequency range, a feasible behavior in systems exhibiting inverse power law relations (Yates, 1992; West, 1999; Aon et al., 2008c; Sasidharan et al., 2012). These results led to the idea that mitochondrial oscillations may play a role as intracellular timekeeper (Aon et al., 2007a; 2008b,c).

Through frequency and amplitude modulation oscillatory dynamics may function as a temporal-encoding signaling mechanism, and ROS-induced ROS release (Zorov et al., 2000; Aon et al., 2003; Zhou et al., 2010) act as an effective coupling and synchronizing mechanism of networked mitochondria because it can exert both local and cell-wide influence (Aon et al., 2004). The present work further adds to this picture in that the inverse relationship between the amplitude and frequency components of the oscillatory H_2O_2 release from mitochondria (**Figure 4**) includes the spatio-temporal functional interdependence between biochemical processes localized in mitochondrial matrix and extra-matrix compartments as depicted in **Figures 1**, **6**. Specifically, the 3D phase space projection of the dynamics of H_2O_2 released as a function of other energetic variables ($\Delta\Psi_m$, succinate) (**Figure 6**) demonstrates the dynamic-functional interrelationships between processes occurring within the same time scale (seconds). This represents a profound insight into the architectural dynamics of complex systems composed of several interrelated dynamic subsystems like the one exemplified by the M-ER model (e.g., membrane potential, SOD activity, respiration, ionic transport). Dynamically speaking, these systems can potentially switch back and forth between low (steady states as fixed point attractors, "simple" limit cycles) and high dimensional dynamic regimens (complex oscillations, chaos) consisting of many degrees of freedom, in this case through slight variations in either ROS production or ROS scavenging. This itinerant dynamic motion (Kaneko and Tsuda, 2003) may confer flexibility to favor the ubiquitous adaptability and evolvability exhibited by organisms in their evolutionary processes. By modulating SOD, cells could have evolved an adaptive compromise between relative constancy ("homeostasis") and the flexibility required under stressful redox/energetic conditions that we have previously redefined as homeodynamics (Lloyd et al., 2001). Unicellular and multicellular organisms match the time dependencies of their internal environments with the periodicities of the external world in the circadian (24 h), ultradian (<24 h), and infradian (>24 h) domains (Lloyd and Murray, 2007; Lloyd and Rossi, 2008; Lloyd et al., 2012). Thus, understanding the mechanisms by which the dynamic elements of complex systems (e.g., biochemical reactions within networks, organelles within cells, coupled oscillators in cell populations) synchronize their function across temporal and length scales becomes a crucial biological problem.

To conclude, we have shown that duplication of antioxidant defenses in different subcellular compartments may represent a powerful strategy in the evolutionary toolkit. Using this strategy cells can control ROS levels and modulate their dynamics with signaling purpose within functionally compatible states.

ACKNOWLEDGMENTS

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Mitochondrial and cellular mechanisms for managing lipid excess

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Current scientific debates center on the impact of lipids and mitochondrial function on diverse aspects of human health, nutrition and disease, among them the association of lipotoxicity with the onset of insulin resistance in skeletal muscle, and with heart dysfunction in obesity and diabetes. Mitochondria play a fundamental role in aging and in prevalent acute or chronic diseases. Lipids are main mitochondrial fuels however these molecules can also behave as uncouplers and inhibitors of oxidative phosphorylation. Knowledge about the functional composition of these contradictory effects and their impact on mitochondrial-cellular energetics/redox status is incomplete. Cells store fatty acids (FAs) as triacylglycerol and package them into cytoplasmic lipid droplets (LDs). New emerging data shows the LD as a highly dynamic storage pool of FAs that can be used for energy reserve. Lipid excess packaging into LDs can be seen as an adaptive response to fulfilling energy supply without hindering mitochondrial or cellular redox status and keeping low concentration of lipotoxic intermediates. Herein we review the mechanisms of action and utilization of lipids by mitochondria reported in liver, heart and skeletal muscle under relevant physiological situations, e.g., exercise. We report on perilipins, a family of proteins that associate with LDs in response to loading of cells with lipids. Evidence showing that in addition to physical contact, mitochondria and LDs exhibit metabolic interactions is presented and discussed. A hypothetical model of channeled lipid utilization by mitochondria is proposed. Direct delivery and channeled processing of lipids in mitochondria could represent a reliable and efficient way to maintain reactive oxygen species (ROS) within levels compatible with signaling while ensuring robust and reliable energy supply.

Keywords: palmitoyl CoA, lipid droplet, perilipin, beta-oxidation, redox environment, energetics, reactive oxygen species

Discovery consists of seeing what everybody has seen and thinking what nobody has thought.

Albert Szent-Gyorgyi

INTRODUCTION

The role of lipids in human health, nutrition, and disease is taking center stage. Several circumstances including hotly debated issues concur to explain this unusual interest. Among them, pressing societal and biomedical issues concerning the epidemic proportions of obesity and related diseases in the United States and its increasing prevalence worldwide. Higher food consumption, decline in physical activity and a progressively aging population are among the social and behavioral roots of this phenomenon. Biologically, it adopts the form of a so-called "metabolic syndrome," a set of comorbidities including upper body obesity, insulin resistance, dyslipidemia, and hypertension that increase the risk for developing type 2 diabetes, coronary artery disease, and stroke (Kok and Brindley, 2012; Schilling and Mann, 2012).

Functional impairments associated with increased circulating levels of lipids and their induced metabolic alterations in fatty acids (FAs) utilization and intracellular signaling, have been broadly termed lipotoxicity (Wende et al., 2012). Current scientific debates concern the association of lipotoxicity with the onset of insulin resistance in skeletal muscle, and with heart dysfunction in obese and diabetic patients.

Mitochondrial function is closely associated with the mounting attention on lipids. One obvious reason is that mitochondria are the main site of lipid degradation. However, the major driving force underlying this association is the fundamental role played by mitochondrial dysfunction in aging and acute or chronic disease conditions such as metabolic disorders (obesity, diabetes), cancer, inflammatory disorders, neurodegeneration, and cardiovascular disease (Akar et al., 2005; Aon et al., 2009; Bugger and Abel, 2010; Camara et al., 2011; Martinez-Outschoorn et al., 2012; Wallace, 2012; Helguera et al., 2013; Cortassa et al., 2014; Rossignol and Frye, 2014).

Cells protect themselves from lipotoxicity or death (Bernardi et al., 2002; Penzo et al., 2002) by either oxidizing FAs or sequestering them as triacylglycerol (TAG) within lipid droplets (LDs) (Greenberg et al., 2011; Walther and Farese, 2012) (**Figure 1**). The ability to store TAG in LDs is evolutionarily conserved and observed in yeast, plants, invertebrates, and vertebrates

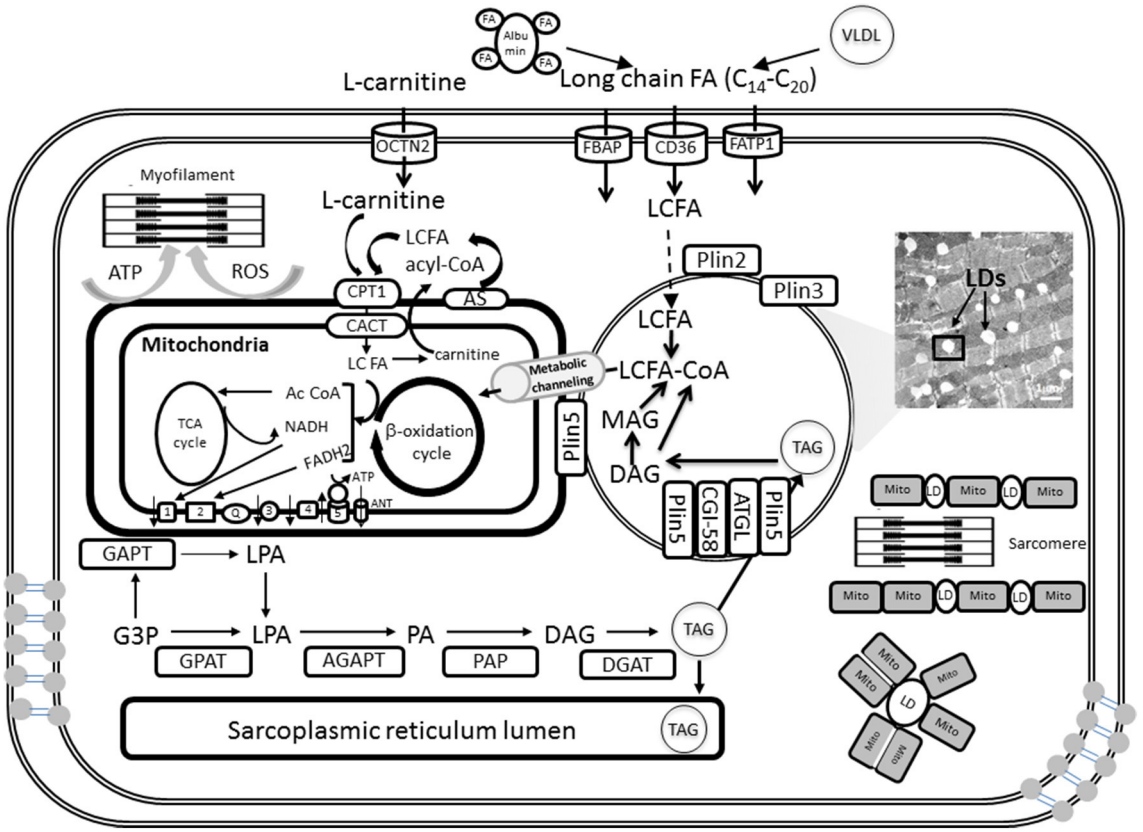

FIGURE 1 | Triglyceride synthesis, storage in lipid droplets, and FA oxidation in cardiomyocyte mitochondria. A detailed explanation of the processes depicted in this figure will be found in sections Lipid Droplets and TAG Metabolism and Fatty Acids and Mitochondrial Function of the main text. LDs can be intercalated with mitochondria or surrounded by them as shown schematically at the right bottom. When mitochondria and LD interact in close contact the scheme suggests that FA degradation and activation occur at the contact sites between both organelles. FA precursors of β-oxidation will be subsequently metabolically channeled to the matrix, and likely β-oxidation, through known pathways (see Section Metabolic Channeling of Lipid Utilization From Close Contacts Between Mitochondria and Lipid Droplets: A Hypothetical-Qualitative Model in the text for more details).

(Walther and Farese, 2012). LDs constitute a highly dynamic FA storage pool that can be used for energy reserve. Recent evidence shows that acute exercise can trigger changes in the dynamics of LD assembly, morphology, localization and mobilization in skeletal muscle, a process regulated by a broad genetic program affecting the spatial and metabolic interaction between mitochondria and LDs. In this process, the exercise-responsive transcriptional coactivator PGC-1α appears to play a key role in coordinating intramuscular LD programming with mitochondrial remodeling (Koves et al., 2013).

There is abundant anecdotal evidence describing close interaction between mitochondria and LD. Early observations indicated that mitochondria are often located near a supply of substrate, or at sites in the cell known to require the ATP generated by the mitochondrion (Lehninger, 1965). Occasional close associations between mitochondria and LDs were found in a variety of tissues such as myocardium, liver, pancreas, and brown adipose. As described by Ghadially (1997):

> "…A single mitochondrion may appear close to, spread out over, or fused to the surface of a small LD, or several mitochondria may be seen surrounding a larger LD. In other instances, the LD may lie in a deep invagination of the mitochondrial envelope, and it is clear that in another plane of sectioning such a droplet could easily be mistaken for a lipid inclusion in the mitochondrion…, particularly if the invaginating membranes are not visualized."

As early as 1958, Palade and Schidlowski suggested that these close associations were meaningful because they "*bring the mitochondrial enzymes into close contact with the lipidic substrate*" (Palade and Schidlowski, 1958, quoted by Ghadially, 1997). Although potential artifacts from sample preparation cannot be ruled out, and that pathologically altered mitochondria can have an influence, when describing lipidic inclusions in mitochondria, Ghadially (1997) wrote:

> "…lipidic inclusions were noted in normal-looking mitochondria with well-formed cristae, where presumably the lipid has a physiological role."

More recent experimental data puts on a more solid ground the idea that there are both physical and metabolic interactions between LD and mitochondria. These interactions appear to be

modulated by relevant physiological situations such as fasting and exercise training. Available evidence also shows that proteins located in the LD surface closely interact with enzymes of the lypolytic cascade modulating FA acid efflux from the droplet.

LIPID DROPLETS AND TAG METABOLISM

TAG is the major form of energy storage that with sterol esters serve as reservoirs of membrane lipid components (Walther and Farese, 2009). In cardiomyocytes TAGs are synthesized by acyltransferases and phosphatases at the sarcoplasmic reticulum and mitochondrial membrane and then packaged into LDs (Walther and Farese, 2009; Singh and Cuervo, 2012; Kienesberger et al., 2013). TAG synthesis is initiated by glycerol-3-phosphate acyltransferases (GPAT) at the mitochondrial and sarcoplasmic reticulum membrane and then completed at the sarcoplasmic reticulum by sn-1-acyl-glycerol-3-phosphate acyltransferase (AGPAT), phosphatidic acid phosphatase (PAP), and sn-1,2-diacylglycerol acyltransferase (DGAT) reactions (Kienesberger et al., 2013) (**Figure 1**). Newly formed TAGs are packaged into cytoplasmic LDs. Thus, lipids are not stored as FAs but as TAGs (triglycerides) produced by a series of esterification reactions that combine three FA molecules with glycerol 3-phosphate; for example, the TAG for palmitate is tripalmitin.

LDs are considered dynamic cellular organelles rather than simple lipid storage depots that, relatively recently, have been implicated in many biological processes (Walther and Farese, 2009, 2012; Greenberg and Coleman, 2011; Singh and Cuervo, 2012). LDs size varies from a diameter of 0.1 μm in yeast to over 100 μm in a white adipocyte. LDs consist of a single protein-decorated phospholipid monolayer that delimits their hydrophobic core from the rest of the cell (Fujimoto and Parton, 2011). The hydrophobic core contains neutral lipids, most notably TAG and sterol esters. The adipose tissue LD has a core predominantly formed by TAG whereas in most cells cholesterol and TAG share the nuclear core of the LD (Singh and Cuervo, 2012). LDs are prominent in many types of mammalian cells, with adipocytes being the most highly specialized for lipid and energy storage. LDs interact with the endoplasmic reticulum and the mitochondria—the two organelles that have been proposed as sites of formation of the autophagosome limiting membrane (Fujimoto et al., 2008; Murphy et al., 2009; Singh and Cuervo, 2012). Such contact zones are also sites of active lipid synthesis enriched in Acyl CoA:diacylglycerol acyltransferase 2 (DGAT2), the major enzyme catalyzing TAG synthesis (Cases et al., 2001; Walther and Farese, 2009).

TAG stored in LDs is catabolized by the sequential hydrolysis of ester bonds between FAs and the glycerol backbone. TAG hydrolysis is a tightly regulated process that involves a complex interaction between lipases and regulatory proteins (Lass et al., 2011). TAG catabolism is performed by a cascade of lipolytic reactions that is initiated by adipose triglyceride lipase (ATGL) producing diacylglycerol (DAG). Hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) complete the lipolytic cascade by sequentially hydrolyzing DAG and monoacylglycerol (MAG), respectively, (**Figure 1**). MAG lipase (MGL) performs the final step in TAG catabolism by hydrolyzing MAGs to glycerol and FAs (Kienesberger et al., 2013). The rate of lipolysis can be dramatically stimulated by adrenergic hormones via activation of protein kinase A (PKA). PKA phosphorylates perilipin and HSL and causes a complex set of events leading to TAG hydrolysis.

The FAs released during TAG catabolism are mainly used for β-oxidation and subsequent ATP synthesis via OxPhos in mitochondria (**Figure 1**; see below: *Fatty Acids and Mitochondrial Function*). In oxidative tissues such as the heart, TAG-derived FAs are utilized as an energy source, but they also serve as signaling molecules as well as building blocks for membranes and complex lipids.

Hepatocytes, heart and skeletal myocytes, adrenocortical cells, enterocytes, and macrophages may all contain large amounts of LDs. Excessive LD accumulation is a hallmark of T2DM, obesity, atherosclerosis, hepatic steatosis, and other metabolic diseases. However, in certain organs like skeletal muscle, intramyocellular triacylglycerol (IMTG) accumulation is not strictly a pathologic phenomenon (see below: *Mitochondria, Lipids and Insulin Resistance*). Lipid content is elevated in red compared with white skeletal muscles and increases in response to habitual exercise in both oxidative and glycolytic fibers. The "athlete paradox" consists of IMTG accumulation observed in endurance-trained athletes that retain insulin sensitivity irrespective of the fact that in some cases IMTGs exceed those measured in sedentary obese or T2DM obese patients (Goodpaster et al., 2001; van Loon et al., 2003; Shaw et al., 2010; Egan and Zierath, 2013; Koves et al., 2013). As with aerobic exercise, both muscle glycogen and IMTG contribute to energy provision during resistance exercise (Koopman et al., 2006).

MITOCHONDRIA AND PERILIPINS

The protein family of perilipins (Plin) is associated with LDs. As scaffolding proteins perilipins affect the spatial and metabolic interactions between LD and mitochondria (**Figure 1**). Development of tissue lipotoxicity and dysfunction is linked to alterations in LD biogenesis and regulation of TAG hydrolysis (Wang and Sztalryd, 2011). Since in response to lipid loading of cells perilipins associate with LDs the role of these proteins is under intense scrutiny.

The Plin protein family, or PAT for perilipin/ADRP/TIP47, is constituted by Plin1 to Plin5, and droplets may contain various combinations of them (Greenberg et al., 2011). Plin1 is the most abundant PAT protein in adipocytes and Plin2 in the liver, where it has been linked to hepatic steatosis. Whereas Plin1 and 4 are limited to adipose tissue, Plin2 and 3 are ubiquitous. Plin1 and 2 are always found in an LD-bound state whereas Plin3 to 5 can be either LD-bound or free in the cytoplasm.

Genetic manipulations aiming at ablating perilipins to infer about their physiological roles and impact on fat deposition have been performed. *Plin1*-null mice are lean and develop systemic insulin resistance as they grow older. *Plin1*-null adipocytes exhibited enhanced rates of constitutive (unstimulated) lipolysis and reduced catecholamine-stimulated lipolysis (Tansey et al., 2001). Together, these data suggested that Plin1 protein enhances catecholamine-stimulated lipolysis and, importantly, that a reduction in Plin1 protein expression is associated with increased constitutive lipolysis, which can promote systemic insulin resistance (Greenberg et al., 2011).

Plin5 is found primarily in oxidative tissues, e.g. skeletal and heart muscles, liver (Bickel et al., 2009). *Plin5* knockout mice lacked detectable LDs in the heart and had significantly reduced myocardial TAG content, an effect that was rescued by lipase inhibition (Kuramoto et al., 2012). The excessive TAG catabolism exhibited by Plin5-deficient hearts was paralleled by increased FA oxidation (FAO) and enhanced ROS levels that led to an age-dependent decline in heart function. Thus, it was suggested that uncontrolled lipolysis and defective TAG storage impair cardiac function through chronic mitochondrial FA overload. Plin5 may regulate LD degradation and the flux of lipolysis-derived FAs to mitochondria for energy production (**Figure 1**) (Kienesberger et al., 2013). Plin5 overexpression in cardiac muscle produced a robust increase in LDs resulting in cardiac steatosis but without major consequences for heart function. This data indicated that Plin5 plays a critical role in droplet formation and stabilization via its regulatory role of lipolysis *in vivo* (Wang et al., 2013). Interestingly, mitochondria in heart tissue from the Plin5 overexpressor appeared to always be distributed in tight clusters around LDs exhibiting a significant increase in size without changes in number as revealed by morphometric analysis (Wang et al., 2013). In skeletal muscle, Plin5 overexpression increased IMCL content without hindering insulin mediated glucose uptake while promoting the expression of genes involved in mitochondrial FAO and fat catabolism (Bosma et al., 2013).

In liver, down-modulation of Plin2 promotes a reduction in hepatic steatosis and increases insulin sensitivity, but a reduction in both Plin2 and Plin3 causes insulin resistance (Greenberg et al., 2011). In the heart, Plin2 does not promote the interaction of mitochondria with LDs, but increased TAG accumulation associated with reduced presence of ATGL in LD and decreased lipolysis (Wang et al., 2011). As the first enzyme from the lipolytic cascade (Zimmermann et al., 2004), the constitutive activity of ATGL is predominantly responsible for basal levels of lipolysis (Greenberg et al., 2011). ATGL overexpression in a cardiomyocyte-specific manner decreased myocardial TAG and lipotoxic intermediates accumulation in type 1 diabetic mice (Pulinilkunnil et al., 2013). This resulted in decreased reliance on FAO, and preserved content of respiratory complexes as well as cardiac function during early stages of diabetes.

Overall, the reported data indicate that reduced expression of perilipins may promote both lipolysis and fat oxidation, resulting in reduced intracellular TAG and adipose mass. On the other hand, excessive lypolysis and defective lipid storage may promote insulin resistance and impaired cardiac function through chronic mitochondrial FA overload. Consequently, lipid storage and utilization appears to be a tightly regulated cellular process.

FATTY ACIDS AND MITOCHONDRIAL FUNCTION

Preservation of the intracellular redox environment (RE) is crucial for vital functions such as division, differentiation, contractile work and survival amongst others (Schafer and Buettner, 2001; Aon et al., 2007, 2009; Brown et al., 2010; Fisher-Wellman and Neufer, 2012; Jeong et al., 2012; Lloyd et al., 2012; Muoio and Neufer, 2012; Aggarwal and Makielski, 2013). Mitochondria are main drivers of the intracellular RE (Aon et al., 2010, 2012; Stanley et al., 2011; Tocchetti et al., 2012; Fisher-Wellman et al., 2013; Kembro et al., 2013) and together with peroxisomes constitute the main subcellular compartments where lipid degradation occurs. Yet, the impact of lipids on mitochondrial redox status and ROS emission, and their links to energetics are not fully elucidated.

FAs are main metabolic fuels in heart and skeletal muscle, and β-oxidation represents their main degradation pathway. The rate of β-oxidation is led by demand since an increase in work rate and ATP utilization leads to faster oxidative phosphorylation (OxPhos) and tricarboxylic acid (TCA) cycle activity. In turn, the decrease in NADH and acetyl-CoA (AcCoA) levels leads to an increase of the β-oxidation flux (Neely et al., 1969; Oram et al., 1973; Eaton et al., 1996a; Eaton, 2002; Lopaschuk et al., 2010).

Lipids are supplied in the form of albumin-bound FAs secreted from adipose tissue or by catabolism of very low density lipoprotein (VLDL) complex by coronary vascular endothelial lipoprotein lipases (**Figure 1**). Long chain FA (LCFA) transport requires carrier proteins in the sarcolemma (FATP1, fatty acid transporter protein 1; FABP, plasma membrane-associated fatty acid-binding protein; LCFAT, long-chain fatty acid transporter; OCTN2, plasma membrane sodium-dependent carnitine transporter; FAT/CD36, fatty acid translocase CD36) and the mitochondria (CPT1, carnitine palmitoyltransferase 1; CACT, carnitine:acylcarnitine translocase).

Upon entry into the cell, LCFA first gets activated by forming thioesters with coenzyme A (CoA), LCFA-CoA, and is either oxidized in the mitochondria via β-oxidation or forms TAG by esterification (**Figure 1**). Subsequently TAGs can be stored in the form of LD. Long-chain FAs are activated on the mitochondrial outer membrane by the long-chain acyl-CoA synthetase but the mitochondrial inner membrane is not permeable to these acyl-CoAs. CPT1 catalyzes the conversion of long-chain acyl CoA to long-chain acylcarnitine, which is subsequently shuttled into the mitochondria (Lopaschuk et al., 2010). Control at the level of CPT1 activity appears to be important in heart and skeletal muscle β-oxidation flux (Awan and Saggerson, 1993; Lopaschuk et al., 1994; Zammit, 1999; Eaton, 2002).

After its formation by CPT1, the long-chain acylcarnitine is translocated across the inner mitochondrial membrane by CACT that involves the exchange of carnitine for acylcarnitine. CACT has extremely high activity in most cell types with active β-oxidation (Ramsay and Tubbs, 1976; Noel et al., 1985; Eaton, 2002). CACT is a critical step in the translocation of FA moieties into the mitochondria, as evidenced by the development of cardiomyopathies and irregular heartbeats in individuals with CACT deficiencies (Lopaschuk et al., 1994, 2010).

In the matrix, acylcarnitine is converted back to acyl CoA and catabolized via β-oxidation. The β-oxidation of activated FAs occurs within the mitochondrial matrix and is catalyzed by the sequential action of four enzyme families (acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl- CoA thiolase), with acyl-CoA dehydrogenase exhibiting different substrate specificity for short-, medium-, long- and very long-chain acyl-CoAs (Kunau et al., 1995; Eaton et al., 1996a; Kerner and Hoppel, 2000). The end product of each cycle of β-oxidation is AcCoA, shortening the LCFA by 2 carbons. Ac CoA then enters the TCA cycle for complete oxidation

rendering reducing equivalents in the form of the electron donors NADH and $FADH_2$ leading to ATP synthesis via OxPhos in the respiratory chain (**Figure 1**). Ultimately, ATP is utilized by the contractile machinery to transduce chemical energy into mechanical work. ROS may also affect contractile performance via signaling or redox modification of sensitive cysteines from, e.g., myosin heavy chain (Canton et al., 2011; Steinberg, 2013).

Besides their metabolic role in the provision of energy, long-chain free FAs exert diverse effects on cellular membranes and on the catalytic activities of many enzymes (Loskovich et al., 2005). FAs play the dual role of uncouplers and inhibitors of mitochondrial respiration (Wojtczak and Schonfeld, 1993) through a protonophoric effect on the inner membrane, and an inhibitory action on the electron transfer chain (Schonfeld and Reiser, 2006; Schonfeld and Wojtczak, 2007, 2008). Additionally, FAs have the potential to drastically alter mitochondrial membranes permeability through opening of the permeability transition pore (Scorrano et al., 2001; Bernardi et al., 2002; Penzo et al., 2002, 2004). Excluded from these effects are the acyl-CoAs that do not exert protonophoric activity and do not uncouple OxPhos because they are unable to cross the inner mitochondrial membrane (Wojtczak, 1976).

Free FAs can act as specific complex I-directed inhibitors (Loskovich et al., 2005; Schonfeld and Wojtczak, 2008), and long-chain acyl-CoAs are known inhibitors of ANT (Pande and Blanchaer, 1971; Lerner et al., 1972; Wojtczak, 1976). The inhibition is of a competitive character (Duszynski and Wojtczak, 1975) and strongly depends on the carbon chain length of the fatty acyl moiety (Morel et al., 1974). Further evidence that FAs, in their anionic form, can be substrates for transport by ANT was given by their inhibitory effect on ATP and ADP exchanges (Wojtczak and Zaluska, 1967; Schonfeld et al., 1996; Klingenberg, 2008). According to the FA cycling model (Skulachev, 1991) undissociated FA molecules can undergo a spontaneous flip-flop from the outer to the inner leaflet of the inner mitochondrial membrane where they release protons because of the alkaline milieu of the matrix. Then, in the form of anions, they are transported back to the external leaflet by ANT; one proton is transferred from the external space to the matrix compartment per molecule of the FA per cycle. In this manner, FAs can lead to energy dissipation through a selective protonophoric action mediated by coupling of transmembrane movement of the fatty acyl anion (via the ANT, uncoupling proteins, UCPs, and/or other inner membrane carriers). These events result in dissipative proton cycling that decreases the proton motive force thereby affecting respiration, ATP synthesis, and ion homeostasis.

Palmitoyl CoA inhibits the ANT independently from β-oxidation, according to more recent evidence obtained in isolated mitochondria from rat liver (Ciapaite et al., 2005) and guinea pig heart (Aon and Cortassa, unpublished) respiring on G/M. In the case of liver mitochondria it was shown that the ANT inhibition induced changes in intra- and extra-mitochondrial ATP concentrations and $\Delta\Psi_m$. This interference with the ANT carrier increased $\Delta\Psi_m$ and the reduction level of coenzyme Q (Bakker et al., 2000) both expected to promote the formation of ROS. Studies further showed that the PCoA-elicited concentration-dependent H_2O_2 formation can be explained by its effect on $\Delta\Psi_m$ that in the presence of 5 μM PCoA showed a 13 mV increase (Ciapaite et al., 2006). The specific action of PCoA on the ANT in the liver (Ciapaite et al., 2006), is in contrast with an apparent multi target effect in the heart (Aon and Cortassa, unpublished). These differences may be given by intrinsic functional differences due to species (rat, guinea pig) or organ specificity, e.g., liver and heart mitochondria. Differences may also be linked to the presence of distinct FA transporters (FATPs or SLC27As) or FA binding proteins (FABPs).

MITOCHONDRIA, LIPIDS, AND INSULIN RESISTANCE

The shift from intermediate values of RE, corresponding to ROS levels compatible with signaling functions (Aon et al., 2010; Cortassa et al., 2014), toward either more reducing or oxidizing conditions is a topic of great potential importance and interest with implications for insulin signaling. Indeed, the association between lipotoxicity and the onset of insulin resistance in skeletal muscle is a hotly debated subject (Muoio and Neufer, 2012). One side posits that it is due to dysfunctional mitochondria with intrinsic deficiencies in OxPhos and deficits in fat oxidation. These impairments impinge on insulin signaling by diverting FAs away from oxidation and toward production of DAGs, ceramide and other toxic lipid species (Lowell and Shulman, 2005; Roden, 2005). The other side of the debate notes that this idea is incompatible with the principles of bioenergetics because mitochondrial respiration is governed by energy demand; intracellular lipids will accumulate whenever FAs supply exceeds the energy needs of the cell. Consequently, they suggest that the etiology of muscle insulin resistance is grounded on the fundamental principles that govern cellular and mitochondrial bioenergetics and the redox stress that is placed on the respiratory system when energy supply persistently outpaces energy demand (Muoio and Neufer, 2012). In agreement with this idea other authors have emphasized that the matching between increased FA availability and oxidative capacity distinguishes the increase in IMTG following endurance training from obesity/diabetic conditions. Chronic exercise training can elicit high oxidative capacity conferred by higher mitochondrial content but not mitochondrial function. Under these conditions, lipid infusion in endurance-trained athletes is able to reduce insulin sensitivity only by 29% as compared to 63% in untrained subjects (Phielix et al., 2012).

Whereas in exercise training IMTG reflects an increased reliance on fats as substrate, in obesity/diabetes will imply accumulation of lipid metabolites [long chain fatty acyl-CoA (LCFA-CoA), DAG, and ceramide] that are responsible for the impairment in insulin action rather than the IMTG pool contained in LDs (Schrauwen et al., 2010; Fisher-Wellman and Neufer, 2012). Apparently, increased concentrations of intramuscular LCFA-CoA and DAG activate PKC, which appears to induce impairments in insulin signaling via serine phosphorylation of the insulin receptor substrate-1. In a model of diet-induced obesity, accumulation of acylcarnitines, as products of incomplete β-oxidation, was shown in skeletal muscle (Koves et al., 2008). These findings led to the idea of a mitochondria-derived signal that couples incomplete β-oxidation with insulin resistance. Chronic elevations of incomplete oxidation intermediates of FAs and branched-chain amino acids (Newgard, 2012) might foster

a mitochondrial microenvironment that is conducive to higher H_2O_2 release from mitochondria with potential to modulate insulin signaling (Fisher-Wellman and Neufer, 2012; Muoio and Neufer, 2012).

The debate about the role of mitochondrial and lipid metabolism at the origin of insulin resistance is highly relevant for the diabetic heart because of its heavy dependence on fats for function (Holloway et al., 2009, 2011). The debate centered on the mitochondrial load-oxidative potential in skeletal muscle, is also relevant for the heart where function is led by energy demand. In fact, lipid accumulation in the heart is largely seen as a mismatch between supply and demand, i.e., lipids amass when supply outpaces demand.

A fundamentally important question still heavily debated is whether or not a shift in substrate preference toward fat oxidation lowers disease risk (Muoio and Neufer, 2012). FAs and glucose are the two major fuels driving heart contraction. In type 2 diabetes and obesity FAO is increased (Lopaschuk, 2002; Carley and Severson, 2005) but our knowledge about the combined effects of hyperglycemia, a hallmark of diabetes, and high FA availability, on metabolism, redox/ROS balance and their impact on heart function is incomplete. Although the healthy heart is flexible regarding fuel selection, in the metabolically challenged diabetic heart by high levels of glucose and fat, the factors contributing to dysfunction and which are beneficial as energy source or redox donors are still unclear. Existing compelling evidence indicates that substrate-driven redox status plays a critical role in cardiac contractile performance in type 2 diabetes where cellular/mitochondrial redox and energetics are altered (see below: *Mitochondrial, Cellular and Organ Mechanisms for Managing Lipid Affluence*) (Anderson et al., 2009a; Tocchetti et al., 2012). Overall, there is no disputing that lipid oxidation confers a metabolic advantage during starvation and exercise, but the role of fuel selection *per se* in defending against metabolic disease needs further investigation.

MITOCHONDRIAL, CELLULAR, AND ORGAN MECHANISMS FOR MANAGING LIPID AFFLUENCE

As important fuels of cellular function it is very well known how FAs are degraded by mitochondria. Yet, the mechanisms by which mitochondria manage lipid excess are largely unknown. The role of β-oxidation *per se* as an underlying cause of obesity-associated glucose intolerance remains a topic of active research and debate (Fisher-Wellman and Neufer, 2012; Muoio and Neufer, 2012). Furthermore, mitochondria play a central role in the development of diabetes and obesity complications (Bugger and Abel, 2010; Sivitz and Yorek, 2010) and their energetic/redox dysfunction is directly involved in the redox imbalance exhibited by the heart (Tocchetti et al., 2012; Frasier et al., 2013) and skeletal muscle (Anderson et al., 2009a).

Mitochondria and lipid oxidation play a predominant role as drivers of the intracellular RE. FAs are a major source of cellular ATP which, in the heart, is synthesized up to two thirds via reducing equivalents (e.g., 24 NADH, 8 $FADH_2$ for palmitate) derived from β-oxidation in mitochondria. The higher energetic budget provided by the saturated FA palmitate (three times higher than from glucose when ATP/mol substrate is considered) in the form of reducing power provides electrons to antioxidant systems and the mitochondria respiratory/energetic machinery. In agreement with the prominent role of lipids on the intracellular redox status, it was shown that Palm determined a transition from oxidized-to-reduced cellular redox status in cardiomyocytes from type-2 diabetic (*db/db*) hearts abating ROS levels drastically (Tocchetti et al., 2012). This effect was coupled to a marked GSH rise both in wild type and *db/db* myocytes. As a consequence of its favorable effect on cellular redox balance, Palm significantly improved isoproterenol-induced contractile reserve in *db/db* cardiomyocytes (Tocchetti et al., 2012).

Keeping a proper cellular/mitochondrial RE is vital for optimal excitation-contraction (EC) coupling as well as energy supply in the heart (Burgoyne et al., 2012; Christians and Benjamin, 2012; Nickel et al., 2013, 2014). Intracellular redox balance affects Ca^{2+} handling by interfering with a wide range of proteins implicated in EC coupling (Fauconnier et al., 2007) including the SR Ca^{2+} release channels [the ryanodine receptors], the SR Ca^{2+} pumps, and the sarcolemmal Na^+/Ca^{2+} exchanger (Zima and Blatter, 2006; Dedkova and Blatter, 2008). Also unknown is whether the mechanisms utilized by mitochondria to deal with lipid excess differ between organs. Important examples are the skeletal and cardiac muscles where β-oxidation predominates due to their lack of *de novo* lipogenesis (Eaton, 2002). Certainly, the organ's functional specificity plays a role. As a matter of fact, skeletal muscle is the largest glycogen storage organ (∼4-fold the capacity of the liver) thus critical for glycemic control as the predominant (∼80%) site of glucose disposal under insulin-stimulated conditions (DeFronzo et al., 1981; Egan and Zierath, 2013). On the other hand, the heart carries out its pump function transducing the chemical energy stored in FAs and glucose into mechanical and electrical energy. At rest, the heart cycles about 6 kg of ATP every day while beating about 100,000 times (Neubauer, 2007). Mitochondria provide the bulk of the ATP needed for cardiac muscle contraction (about two thirds) and sarcolemmal and sarcoplasmic ion transport (one third), responsible for the Ca^{2+} transients and electrical activity in cardiac cells (Solaini and Harris, 2005; Cortassa et al., 2009; Nickel et al., 2013).

The far higher amounts of O_2 processed by the heart on a specific basis with respect to, e.g., brain and skeletal muscle (Rolfe and Brown, 1997), and its continuous activity, make this organ susceptible to oxidative damage (Burgoyne et al., 2012; Christians and Benjamin, 2012). As a matter of fact, myocardial function and the ability of the heart to tolerate stress decline with age (Lakatta and Sollott, 2002). Although the mechanisms contributing to age-related alterations in myocardial function are not fully understood, mitochondrial dysfunction, oxidative stress and the accumulation of oxidant-induced damage are major factors (Fannin et al., 1999; Suh et al., 2003; Judge et al., 2005).

Defects in mitochondrial FA β-oxidation lead to several well-known metabolic disorders, such as Reye syndrome, cardiomyopathy and sudden infant death syndrome (Roe and Ding, 2001; Yang et al., 2001). The maintenance of high levels of mitochondrial β-oxidation could reduce the excessive fat accumulation and storage leading to human obesity. Lipid overload involving TAG accumulation in non-adipose tissues characterizes disorders such as hyperlipidemia and lipodystrophies, heart dysfunction, liver

disease, in both humans and in animal models of obesity and diabetes.

It is becoming increasingly clear that adequate regulation of TAG metabolism in different organs is critical for both energy metabolism and function. Liver and heart respond to the massive influx of lipids from blood by up regulating LD biogenesis, as a mechanism of defense against the toxicity of FAs, which upon esterification get converted into TAG and stored into LD (Lass et al., 2011). Failure to do so in the liver originates pathogenic conditions such as steatosis and steatohepatitis (Greenberg et al., 2011). The lipid excess situation is also relevant for heart function in T2DM where FAs are preferred fuels (Lopaschuk et al., 2010). However, under acute, non-chronic, conditions FAs can exhibit advantageous actions, especially in the heart under diabetic conditions (Tocchetti et al., 2012). Cellular TAG accumulation in LDs may be beneficial rather than detrimental because it diverts FAs from pathways leading to cytotoxicity thus serving as a buffer against lipotoxicity (Listenberger et al., 2003).

From the examples and arguments above, it is clear that lipids have a considerable impact on many cellular processes, including mitochondria. This impact influences the functional outcome of several organs such as the liver, skeletal and cardiac muscles. Deregulation of lipid metabolism produces overload that is at the origin or as an aggravating consequence of many diseases. Consequently, the fundamental as well as practical importance of unraveling the mechanisms by which mitochondria handle lipids excess cannot be overstated. First, at the most basic level, we do not know enough about lipids action on mitochondrial energetic and redox functions. Lipids can act both as uncouplers and inhibitors of OxPhos (Wojtczak and Schonfeld, 1993; Bernardi et al., 2002), and the consequences of these contradictory effects on mitochondrial energetic, redox and signaling functions are just starting to be unraveled (Schonfeld and Wojtczak, 2008). Second, besides being the main site of lipid degradation, mitochondria may be actively modulating the balance between lipid storage and utilization.

In the following sections we explore some of the new emerging mechanisms of lipid storage and utilization by mitochondria at the organelle, cellular and organ level in different physiological settings.

CLOSE CONTACT MITOCHONDRIA-LIPID DROPLET

Regular exercise and physical activity are considered cornerstones in the prevention, management, and treatment of numerous chronic conditions, including hypertension, coronary heart disease, obesity, T2DM, and age-related muscle wasting (sarcopenia) (Haskell et al., 2007; Colberg et al., 2010; Egan and Zierath, 2013).

Exercise training enhances mitochondrial biogenesis and performance in skeletal muscle (Irrcher et al., 2003), but not in the heart (Li et al., 2011). Whether the same is true in T2DM hearts is unclear. In electron micrographs LDs can be easily detected in type 2 diabetic (*db/db*) (Boudina et al., 2007) or *ob/ob* (Ge et al., 2012) but not in WT mice hearts. In cells LDs can be readily visualized using the fluorescent FA analog (dodecanoic acid) BODIPY that labels neutral lipids in cytoplasmic droplets (Walther and Farese, 2012).

The occurrence of close contact between mitochondria and LD in the heart is remarkable because of its dependence on mitochondrial energetics preferentially fueled by FAs. More noteworthy though is the fact that these close contacts occur in the T2DM heart, where the dependence on fat fueling is even more prominent (Lopaschuk, 2002; Bugger and Abel, 2010). Interestingly, Plin5 overexpression in heart tissue rendered tight mitochondrial clusters around LDs with mitochondria significantly larger but not higher in number (Wang et al., 2013). The same authors proposed that Plin5 could play a regulatory role in the FA flux from LDs to mitochondria under conditions of increased cellular FA influx (Wang and Sztalryd, 2011). These data also suggest that Plin5 with its role of favoring LD accumulation may act to keep the intracellular levels of FA metabolites (e.g., DAG, ceramide) below lipotoxic amounts (see below: *Metabolic Channeling of Lipid Utilization From Close Contacts Between Mitochondria and Lipid Droplets: A Hypothetical-Qualitative Model*).

In skeletal muscle IMTG accumulates and is actively utilized during exercise (Shaw et al., 2010; Egan and Zierath, 2013; Koves et al., 2013). Endurance exercise training increases mitochondrial content (by size not numbers) for men and women but healthy active women have higher IMTG accumulation compared with men due to greater number rather than size of LDs (Tarnopolsky et al., 2007). Interestingly, this study also reported an increase in the physical contact between mitochondria and IMTGs following endurance exercise training. Rates of whole body fat oxidation and IMTG utilization are determined by factors such as diet, intensity and duration of exercise, and fitness. During acute exercise, the contribution of various metabolic pathways to energy provision is determined by the relative intensity and absolute power output of the exercise bout (Egan and Zierath, 2013). The rate of ATP demand and energy expenditure is determined by the absolute power output whereas the relative exercise intensity influences the relative contributions of carbohydrate oxidation and lipid sources, and circulating (extramuscular) and intramuscular fuel stores, to energy provision. As exercise intensity increases, muscle utilization of circulating free FAs slightly declines, whereas utilization of circulating glucose increases progressively up to near-maximal intensities (van Loon et al., 2001).

IMTG breakdown occurs primarily via HSL and ATGL (Watt and Spriet, 2010). Although IMTGs constitute only a small fraction (∼1–2%) of whole-body lipid stores they represent an important fuel source during prolonged (>90 min) but moderate intensity exercise. IMTGs can provide ∼25% of total energy however their contribution decreases at either higher or lower intensities of exercise (Romijn et al., 1993; van Loon et al., 2001). Maximal rates of fat oxidation occur at moderate exercise intensities (∼60% VO_2 max) (Shaw et al., 2010; Egan and Zierath, 2013). At low-to-moderate exercise intensity, the primary substrates fueling skeletal muscle are glucose, derived from hepatic glycogenolysis (or gluconeogenesis) or oral ingestion, and free FAs released by adipose tissue lipolysis. Prolonged exercise (>60 min) at a fixed intensity increases the energy contribution from lipid oxidation (Egan and Zierath, 2013). IMTG stores can be reduced by ∼60% following exercise, predominantly in type I muscle fibers (van Loon et al., 2003;

Stellingwerff et al., 2007; Shaw et al., 2010; Egan and Zierath, 2013).

Lipophagy, i.e., the turnover of LDs by autophagy, may occur due to random sequestration of cytosolic material by "in bulk" autophagy. However, when lipophagy is activated in response to a lipid challenge or prolonged starvation, a switch toward the preferential sequestration of LD seems to happen, supporting some level of selectivity in this process (Singh et al., 2009). We suggest that this may also be the case for close contacts mitochondria-LD, and that energy demand may be a main elicitor of the interaction between these two organelles. Consonant with this idea, it has been proposed that LDs assembly in skeletal muscle under exercise training would improve the management of high FA influx enabling a more precisely regulated trafficking of substrate to and from IMTG thus contributing to optimal mitochondrial performance and metabolic flexibility (Koves et al., 2013).

LIPOTOXICITY AND LD ACCUMULATION DYNAMICS

In pathologic states lipotoxicity may occur over time, despite TAG accumulation, when either the cellular capacity for TAG storage is exceeded or when triglyceride pools are hydrolyzed, resulting in increased cellular free FA levels. Thus, the duration and extent of lipid overload may determine if a cell is protected or damaged. Whether mitochondrial energy/redox status can alter the balance LD formation and utilization in the short-term is a question that has not been hitherto addressed.

Studies performed with non-invasive spectroscopic techniques have shown elevated IMCL triglyceride content in the left ventricle (i.e., LV steatosis) of obese and T2DM patients (McGavock et al., 2007; Rijzewijk et al., 2008) but its association with early diastolic dysfunction leading to subsequent systolic dysfunction remains controversial (Anderson et al., 2009b; Lopaschuk et al., 2010). Again, lipids through accumulation of triglycerides are at the center of the controversy. In skeletal (Liu et al., 2007) and cardiac (Ussher et al., 2009) muscle, IMCL accumulation as a result of diet-induced obesity is not at all pathogenic, but may even be protective against obesity-associated maladies.

Previous reports have linked ROS-mediated mitochondrial dysfunction to DAG and ceramide, two main products of lipid degradation (Coen and Goodpaster, 2012). Lipid channeling to mitochondria may represent a mechanism by which concentration build-up of these intermediaries is avoided, especially under high energy demand. Based on these premises, we suggest that temporary lipid storage in LDs does not necessarily represent pathophysiological behavior. On the contrary, it may embody an adaptive response, at least in the short-term thus representing an adaptive strategy of lipids utilization ensuring energy supply without affecting neither mitochondrial nor cellular redox status.

REDOX OPTIMIZED ROS BALANCE AND MITOCHONDRIAL REDOX AND ENERGETICS

Lipid metabolites can damage the respiratory chain leading to impaired energetic transition in mitochondria through their dual effect as uncouplers and inhibitors (Wojtczak and Schonfeld, 1993). Impairment of the key state 4→3 energetic transition can occur via inhibition of ANT or ATPsynthase thereby producing a continuous release of ROS irrespective of ADP addition (Tocchetti et al., 2012).

Mitochondria are a main source of ROS but can also be their target. The RE is a major driving force of the crucial energy-redox link of mitochondrial function (Cortassa et al., 2014). The mitochondrial RE depends on the intrinsic redox potential and instantaneous reducing capacity of this organelle as well as its response to the cytoplasmic redox status (Aon et al., 2010; Kembro et al., 2013). In this context, Redox-Optimized ROS Balance (R-ORB) provides a useful conceptual framework to rationalize many results described in the present review. One of the main R-ORB postulates is that ROS efflux from mitochondria will attain a minimum at intermediate values of RE, when VO_2 reaches a maximum following ADP stimulation (**Figure 2**) (Cortassa et al., 2014). Under state 3 respiration, glutathione and thioredoxin systems are essential for minimizing ROS release from mitochondria (Aon et al., 2010, 2012; Stanley et al., 2011; Kudin et al., 2012; Cortassa et al., 2014). In excess, lipid precursors of β-oxidation can promote mitochondrial uncoupling and oxidized redox status (Aon and Cortassa, unpublished). In more oxidized RE, away from the optimum (intermediate) RE compatible with minimal ROS, antioxidant systems become overwhelmed leading to pathological ROS overflow (Aon et al., 2010; Cortassa et al., 2014).

Mitochondria function in more oxidative environments in chronic diseases (Tocchetti et al., 2012). Thus, it becomes fundamental to understand how oxidative stress influences the dependence of ROS emission on respiration (Cortassa et al., 2014). When oxidant challenged, mitochondria displayed H_2O_2 emission levels 2-fold higher than controls, and exhibited lower respiration (**Figure 2**). Oxidative stress shifted redox balance toward the more oxidized range where the sensitivity of the ROS efflux to the RE decreases more drastically in state 4 than in state 3 respiration. A 50% decrease in reduced glutathione (GSH) was mainly responsible for the shift of the RE to a more oxidized state (Cortassa et al., 2014).

METABOLIC CHANNELING OF LIPID UTILIZATION FROM CLOSE CONTACTS BETWEEN MITOCHONDRIA AND LIPID DROPLETS: A HYPOTHETICAL-QUALITATIVE MODEL

Recent evidence supports physical and metabolic interactions between LDs and mitochondria mediated by the scaffolding protein Plin 5 (Wang and Sztalryd, 2011; Wang et al., 2011; Koves et al., 2013). Wang and collaborators observed that Plin5-overexpressing cells show decreased LD hydrolysis and palmitate β-oxidation when compared with controls. Instead, palmitate increasingly incorporated into TAGs under basal conditions whereas in protein kinase A-stimulated state LD hydrolysis inhibition was removed and FAs released for β-oxidation. These results suggested that Plin5 regulates LD hydrolysis and controls local FA flux to protect mitochondria against excessive exposure to FA (Wang and Sztalryd, 2011). All these observations are in agreement with the relatively recent realization that the LD proteome is highly dynamic and more complex than previously thought. The LD proteome contains key components of the fat mobilization system and proteins that suggest LD interactions with a variety of cell organelles, including the mitochondria (Beller et al., 2010).

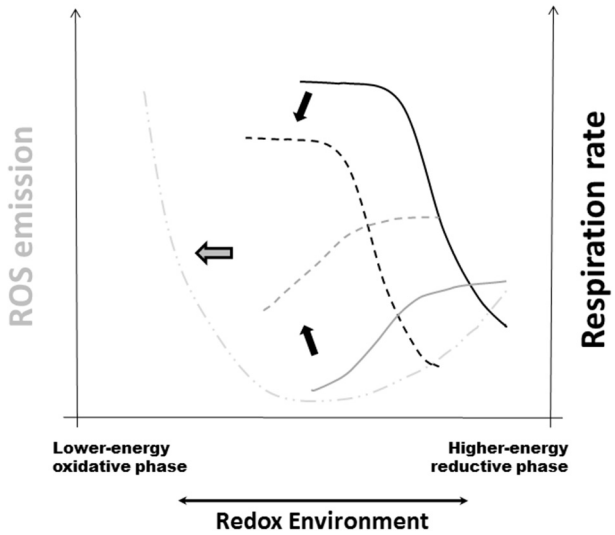

FIGURE 2 | Redox-Optimized ROS Balance and the effect of oxidative stress on mitochondrial respiration, H_2O_2 emission, and the RE. R-ORB postulates that ROS levels (as the net result of production and scavenging) depend on the intra-cellular and -mitochondrial redox environment (RE). It also proposes that there is a minimum level of ROS emission when mitochondria maximize their energetic output. Under high energy demand, and despite large respiratory rates, ROS emission levels will be kept to a minimum by ROS scavenging systems (Stanley et al., 2011; Aon et al., 2012). Oxidative stress can happen at either extreme of RE, either highly reduced or highly oxidized, but governed by different mechanisms (Aon et al., 2010; Kembro et al., 2014). The plot displays schematically the summary of the response of respiration (black traces) and ROS emission in stressed mitochondria (gray traces) plus further addition of the uncoupler FCCP (dashed-dotted line). Continuous lines correspond to the absence of stress whereas dashed lines belong to mitochondria under stressed conditions (Cortassa et al., 2014). Black arrows indicate the direction of change in VO_2 and ROS elicited by stress. Notice the shift toward more oxidized RE in the curves corresponding to stressful conditions. The thick gray arrow pointing to the left denotes pathological conditions arising, e.g., from chronic diseases, where severe stress will affect both energetic (e.g., $\Delta\Psi_m$, ADP consumption) and redox [e.g., NAD(P)H, GSH, Trx] functions thus increased mitochondrial ROS emission and higher cytoplasmic ROS levels. Reprinted from Cortassa et al. (2014).

Based on the premise of metabolic links extending beyond physical contact between mitochondria and LDs, we propose a model of metabolic channeling for lipid utilization by mitochondria. According to our model, metabolic channeling represents a way mitochondria can manage lipid affluence in an energetically and redox-controlled fashion. Qualitatively, the lipid utilization channeling model postulates that after TAG degradation, lipids are directly delivered for activation, transport and β-oxidation from the LD to the mitochondrion at the contact site (**Figure 1**). The model also proposes that β-oxidation may also happen metabolically channeled through the enzymatic components of the lipid degradation pathway organized as a multienzyme complex (Eaton, 2002).

From a structural standpoint, the model is based on direct and close contact between LDs and mitochondria involving their recruitment and surrounding of the LD. The model also postulates membrane fusion-mediated reorganization of intra-mitochondrial membrane and molecular components (Walther and Farese, 2009) as well as lipids segregation within the droplet (Fujimoto and Parton, 2011).

Biochemically, the pathway of long-chain FAO to AcCoA is one of the longest unbranched pathways in metabolism, containing 27 intermediates between palmitoyl-CoA and AcCoA (Eaton, 2002). That the enzymes of β-oxidation may be organized into a multienzyme complex was suggested long ago. In these biomolecular assemblies, sequential catalytic reactions proceed via transfer of the intermediates between individual component enzymes, precluding their diffusion into the bulk aqueous medium, thus "metabolically channeled" (Welch, 1977; Sumegi et al., 1991).

An earlier proposal of metabolic channeling in β-oxidation was based on the detection of low concentrations of intermediates (Garland et al., 1965) and the observation that β-oxidation intermediates that accumulate behaved more like products than intermediates (Stewart et al., 1973; Stanley and Tubbs, 1974, 1975; Eaton et al., 1994, 1996a,b, 1999). This led to the "leaky hosepipe" model for the control of β-oxidation flux (Stewart et al., 1973; Stanley and Tubbs, 1974, 1975) in which channeling of a small, quickly turning-over pool of intermediates was implied (see Eaton, 2002 for a review).

Some aspects of the structural basis for a channeling mechanism in β-oxidation have been described (Ishikawa et al., 2004). Evidence in support of a multifunctional FAO complex within mitochondria, physically associated with respiratory chain supercomplexes that favor metabolic channeling, has been recently reported (Wang et al., 2010). Functionally, the direct delivery of lipids at contact sites, and their channeled processing will avoid elevation of their concentration, thus ruling out the potential inhibitory as well as uncoupling action of FAs (Wojtczak and Schonfeld, 1993). The latter will ensure a reliable and efficient energy supply.

CONCLUDING REMARKS

Mitochondria, cells and organs have developed mechanisms that allow managing heavy influx of FAs within functionally reliable limits. The LD as a dynamic storage of FAs can also be seen as a protective mechanism employed by cells to avoid excessive intracellular concentration of FAs thus hindering their potential deleterious effects on mitochondrial function. The tight and reciprocal regulation of lipid storage and utilization is evidenced by genetic manipulation of perilipins indicating that their reduced expression leads to increased lipid oxidation and reduced accumulation of intracellular fat and adipose mass. On the other hand, however, excessive lipolysis and defective lipid storage promotes insulin resistance through mitochondrial FA overload and ROS overflow.

Preservation of the intracellular RE is crucial for vital functions. Mitochondria play a decisive role as the organelle that specifically handles the highest amounts of oxygen processed by the organism thus prone not only to be the source but also the target of oxidative stress. Mitochondrial function needs to sustain energy supply reliably while releasing ROS levels compatible with signaling. However, lipids can derail both of these critical functions. Consequently, the hypothetical lipid utilization channeling model we are proposing herein satisfies the fundamentals

of cellular and mitochondrial energetics and redox. In principle, diversion of excess lipids to LDs can be an effective cytoplasmic mechanism for "sequestering" FAs thereby helping to keep low concentration of lipotoxic intermediates resulting from lipid oxidation. Functionally, direct delivery and channeled processing of lipids in mitochondria could represent a reliable and efficient way to ensure energy supply and redox control. Such a mechanism would avoid exceeding the lipid storage capacity thus becoming crucial for skeletal muscle or heart subjected to high workload, and therefore, heavy influx of FAs.

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Bcl-xL in neuroprotection and plasticity

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Accepted features of neurodegenerative disease include mitochondrial and protein folding dysfunction and activation of pro-death factors. Neurons that experience high metabolic demand or those found in organisms with genetic mutations in proteins that control cell stress may be more susceptible to aging and neurodegenerative disease. In neurons, events that normally promote growth, synapse formation, and plasticity are also often deployed to control neurotoxicity. Such protective strategies are coordinated by master stress-fighting proteins. One such specialized protein is the anti-cell death Bcl-2 family member Bcl-xL, whose myriad death-protecting functions include enhancement of bioenergetic efficiency, prevention of mitochondrial permeability transition channel activity, protection from mitochondrial outer membrane permeabilization (MOMP) to pro-apoptotic factors, and improvement in the rate of vesicular trafficking. Synapse formation and normal neuronal activity provide protection from neuronal death. Therefore, Bcl-xL brings about synapse formation as a neuroprotective strategy. In this review we will consider how this multi-functional master regulator protein uses many strategies to enhance synaptic and neuronal function and thus counteracts neurodegenerative stimuli.

Keywords: apoptosis, mitochondria, synaptic plasticity, calcium, permeability transition pore

INTRODUCTION

Recent discoveries have shed light on the increasingly complex and varied activities of Bcl-2 family proteins. Discovery of novel roles for these proteins in the nervous system suggests that protection from cell death is a complex operation that is integrated with other important cellular pathways. The anti-apoptotic protein Bcl-xL has been found to regulate mitochondrial bioenergetic efficiency, synaptic transmitter release, synaptic vesicle recycling and neurite growth (**Figure 1**) in addition to its canonical role in preventing the activity of pro-apoptotic proteins. Although it is tempting to assign a primary function, such as regulation of metabolism or cell death (survival), none of these roles appears obviously more important than any other. In the nervous system, proteins that have evolved to regulate differentiation, synaptic connectivity and survival during development are used in adult life to increase cellular efficiency and to enhance synaptic plasticity. Bcl-xL expression is maintained throughout life, perhaps to make new synaptic connections and to potentiate neuronal connections. These features may help in neurite or synapse competition between stronger and weaker members, promoting neuronal soma and process "survival." If the supposition is true, then perhaps Bcl-xL is one of a number of proteins that plays a central role not only in cellular differentiation but also in functional adaptation during adult life in order to maintain cell usefulness to prevent degeneration.

Following that line of reasoning, one can posit that neurodegenerative disease is related to at least two phenomena: Disruption of the roles of protective multifunctional proteins or passive neglect. For example, neuronal overuse over a lifetime may compromise the metabolic and protein milieu, causing a vicious cycle of damage and increased inefficiency. In contrast, if synaptic connections become inactive over time, those synapses may undergo structural and metabolic remodeling leading to decreased metabolic and neuronal network efficiency. Overuse and underuse may both, therefore, result in degeneration, especially in inherently vulnerable neuronal populations.

In this review, we will look at the role of Bcl-xL from the point of view of its multiple closely connected roles in metabolism, growth, synaptic connectivity and plasticity. We will reflect on how increases in metabolic efficiency may underlie effective synaptic and growth enhancement. We will then raise the possibility that a decrease in activity may be correlated with long term depression of neuronal synaptic and electrical activities followed by synapse deconstruction, axonal retraction and eventual somatic loss.

Bcl-xL REGULATES MITOCHONDRIA DURING NEUROTRANSMITTER RELEASE

Many of the features of synaptic transmission can be enhanced over the short and long term (Bliss and Collingridge, 1993). These include changes in presynaptic calcium levels, changes in vesicle numbers and probability of release, and alterations in postsynaptic receptor numbers and function. Such phenomena are

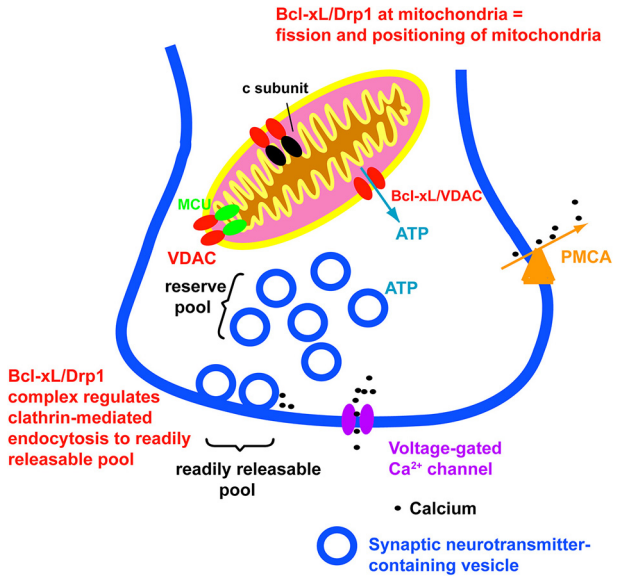

FIGURE 1 | Bcl-xL affects aspects of normal neuronal function and plasticity. These include release of ATP from mitochondria, closing of a leak conductance in the mitochondrial inner membrane, modulation of mPTP, and regulation of synaptic vesicle recycling. Not illustrated is the canonical role of Bcl-xL in prevention of activation of pro-apoptotic factors.

regulated by mitochondria which provide energy in the form of ATP and buffer calcium at active synapses. Mitochondria, therefore, are required for normal synaptic transmission at high frequencies (David and Barrett, 2003; Ivannikov et al., 2013). In addition, calcium uptake and re-release by mitochondria during neurotransmission regulate short term plasticity (Blaustein et al., 1978; Friel and Tsien, 1994; Tang and Zucker, 1997; Mochida, 2011; Lee et al., 2012; Wan et al., 2012) and calcium enhances enzymatic activity of several TCA cycle enzymes (Wan et al., 1989).

Mitochondrial bioenergetics may be altered acutely in synapses that have undergone conditioning, providing for enhanced oxidative competence (Nguyen et al., 1997). Unexpectedly, the Bcl-2 family proteins that are known to regulate apoptosis through their actions at mitochondrial membranes have also been newly identified as regulators of synaptic activity. Thus, the actions of Bcl-xL—an anti-cell death Bcl-2 family member—at mitochondria, position it to influence learning, memory, and alterations in behavior (Jonas, 2006). The ways in which Bcl-xL enhances the bioenergetic functioning of a neuron and the relationship of this to alterations in other cellular and synaptic activities will be addressed.

NEURONAL ACTIVITY INFLUENCES MITOCHONDRIAL CALCIUM UPTAKE AND THE STRENGTH OF SUBSEQUENT SYNAPTIC EVENTS

One key feature of synaptic transmission is marked calcium influx through glutamate receptors and voltage gated calcium channels (Fioravante and Regehr, 2011; Morris, 2013; Simms and Zamponi, 2014). After calcium entry, calcium clearance from the cytosol occurs through the actions of calcium ATPases at the plasma membrane and by buffering through uptake by intracellular stores including ER and mitochondria (Rizzuto et al., 2012; Lopreiato et al., 2014); these processes reset the normal low calcium levels present in resting synapses. The calcium that is buffered by intracellular stores is eventually re-released, providing for residual calcium in synaptic endings. Residual calcium may be necessary for certain forms of synaptic plasticity (Neher and Sakaba, 2008; Mochida, 2011). Mitochondria regulate cytosolic levels of calcium and the release of metabolites through an intricate system involving several ion channels. The machinery that takes up calcium is very important to energy production, neuronal excitability and synaptic plasticity. The discovery of the molecular substrate for the calcium uniporter ion channel (MCU) at the mitochondrial inner membrane has generated increasing interest in mechanisms of calcium management within the neuronal cell body or synapse (Kirichok et al., 2004; Baughman et al., 2011; De Stefani et al., 2011; Mallilankaraman et al., 2012b). Additional isoforms of MCU and its helper MICU that confer tissue specificity and other behaviors have now been identified (Mallilankaraman et al., 2012a; Raffaello et al., 2013). Although not completely understood yet, new findings portend that sites of calcium adjustment will determine activity-dependent energy responses of mitochondria (De Stefani and Rizzuto, 2014) that could be extremely relevant for synaptic plasticity and repair.

CALCIUM INFLUX INTO ACTIVE NEURONS ALTERS ATP SYNTHESIS AND RELEASE BY MITOCHONDRIA

Mitochondria function symbiotically with eukaryotic cells to provide energy in the form of ATP. They take up substrates from the cytosol in the form of products of glycolysis, lipid and protein metabolism. One of the main products of glycolysis, pyruvate, is acted on by pyruvate dehydrogenase to form acetyl co-enzyme A which enters the TCA cycle. Turns of the TCA cycle synthesize NADH and FADH2 that donate their electrons to the electron transport chain. The energy of the bonds of NADH and FADH2 is used to pump H+ ions out of the matrix, creating a proton motive force that in turn drives the ATP synthase. Upon kinetic repositioning of the ATP synthase rotor, ATP is synthesized from ADP and Pi (Watt et al., 2010). The machinery required for ADP influx into the matrix including the outer membrane ion channel VDAC and the adenine nucleotide transporter (ANT) in the inner membrane are intimately linked to that of the ATP synthase (Chen et al., 2004).

Calcium regulates TCA cycle enzymes (Denton, 2009) and enzymes in the electron transport chain (Gellerich et al., 2010) to speed the process of ATP synthesis. As cytosolic calcium increases during neuronal activity, mitochondrial TCA cycle enzymatic activity is enhanced, increasing the levels of mitochondrial NADH and ATP. Calcium influx into mitochondria enhances the proportion of pyruvate dehydrogenase complex in its active, dephosphorylated form. Calcium also increases the ability of isocitrate dehydrogenase and oxoglutarate dehydrogenase to bind their substrates. The result is regulation of mitochondrial TCA enzymes by cytosolic calcium or "external regulation" as opposed to "internal regulation" that is caused by changes in NAD/NADH ratio and the ratio of ADP/ATP in the mitochondrial matrix. In neurons,

increases in cytosolic calcium arise from depolarization of the plasma membrane with resultant increase in permeability to calcium through glutamate receptors and voltage gated calcium channels. In addition, mitochondria receive calcium from the ER which partners with mitochondria to respond to cytosolic calcium loads. Elevated cytosolic calcium enhances the release of calcium from IP3 receptors directly into mitochondria within a tightly coupled space between the two organelles (Rizzuto et al., 2012). Oscillatory calcium release from ER provided by IP3 receptors causes influx of calcium into the mitochondria and drives TCA cycle enzymes even more effectively than sustained elevations of mitochondrial calcium (Hajnoczky et al., 2000), most likely because sustained calcium allows re-equilibration of the signal in the matrix.

The interaction between neuronal activity, calcium influx into mitochondria and energy generation was clarified recently in a study in Drosophila neuromuscular junction (Chouhan et al., 2012). Using a complex set of imaging techniques including genetically encoded calcium/pH indicators, it was shown that neuronal activity enhanced calcium uptake by mitochondria, increased mitochondrial NAD(P)H levels and hyperpolarized the mitochondrial inner membrane potential. These events were inhibited by pharmacological agents that blocked mitochondrial calcium uptake. Interestingly, the level of cytosolic calcium remained similar in different neurons despite their very different firing rates, suggesting that a certain level of cytosolic calcium is optimum for energy production during activity. This specific cytosolic calcium level is most likely achieved by an ideal combination of calcium buffering inside, and extrusion from, the nerve ending. An exciting implication of these novel findings is that in different cell types, changes in cytosolic calcium levels could theoretically produce plasticity in mitochondrial responses to adjust to changes in energy demands in synapses undergoing long term changes in strength.

Another type of mitochondrial management of cytosolic calcium was observed in the large mammalian brain stem presynaptic terminals of the Calyx of Held. In this study, mitochondria were found to remove calcium during rises in cytosolic calcium produced by calcium influx though voltage gated channels. Mitochondrial calcium uptake occurred early in the stimulation paradigm, and dampened the overall rise in calcium in the presynaptic terminal that occurred in the absence of functional mitochondria. The effect of this was to limit neurotransmission produced by a short train of stimuli. The authors concluded that mitochondrial calcium buffering served to prevent synaptic depression by attenuating vesicle depletion and allowing for continued effective synaptic transmission during a train of action potentials (Billups and Forsythe, 2002).

In addition to calcium buffering, acute changes in mitochondrial membrane activity were found to be necessary for short term synaptic potentiation in squid presynaptic terminal. Through the use of a double-barreled patch pipette (Jonas et al., 1997) recordings were made inside the presynaptic terminal on mitochondria both at rest and during and after intense synaptic stimulation (Jonas et al., 1999). In control recordings within the resting presynaptic terminal, the conductance of mitochondrial membranes was found to be low. In contrast, during high frequency electrical stimulation of the presynaptic nerve, a large increase in mitochondrial membrane activity occurred (Jonas et al., 1999). The delay in onset of the mitochondrial activity and the persistence of the mitochondrial activity after stimulation implied that mitochondrial channel activity was not simultaneous with plasma membrane channel activity. This suggested that the increase in activity depended on an intracellular second messenger, most likely calcium (Csordas et al., 2012). In keeping with this, mitochondrial activity was abrogated by removing calcium from the bathing medium during stimulation, demonstrating that the evoked mitochondrial membrane channel activity was dependent on calcium influx into the terminal and into mitochondria (Jonas et al., 1999). The uncoupler FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), which depolarizes mitochondria and prevents calcium uptake, eliminated the mitochondrial channel activity, presumably because calcium uptake into mitochondria during nerve stimulation was abrogated. In keeping with the role for this activity in short term plasticity, FCCP application eliminated posttetanic potentiation of the synapse following high frequency nerve stimulation.

Bcl-xL REGULATES MITOCHONDRIAL ATP RELEASE DURING SYNAPTIC ACTIVITY

The above studies raised the possibility that opening of mitochondrial channels produces release of metabolites and/or calcium itself in response to calcium influx. After calcium influx in the synapse, an inner membrane calcium sensitive channel might also be in contact with an outer mitochondrial membrane channel in order to transfer ions and ATP across the outer as well as the inner membrane. The most ubiquitous outer membrane channel is VDAC, which releases metabolites including ATP into the cytosol (Vander Heiden et al., 2001; Gottlieb et al., 2002). In addition to ATP release, VDAC regulates the uptake of ADP and other metabolites during normal and pathological cell activities (Rostovtseva and Colombini, 1997; Mannella and Kinnally, 2008; Maldonado and Lemasters, 2012) and is regulated by cytoskeletal elements, in particular tubulin in its dimeric form (Rostovtseva and Bezrukov, 2012).

The Bcl-2 family also regulates outer membrane channel activity. During programmed cell death, mitochondrial outer membrane permeabilization (MOMP) (Green and Kroemer, 2004; Dejean et al., 2005; Adams and Cory, 2007), is produced by formation of large outer membrane pores comprised of activated oligomerized pro-apoptotic Bax proteins, aided by other pro-apoptotic moieties (Antonsson et al., 2000; Dejean et al., 2005; Kim et al., 2006). In their canonical role, the anti-apoptotic Bcl-2 family proteins such as Bcl-xL protect cells against MOMP by interacting with, and preventing the activities of, the pro-apoptotic family members (Galonek and Hardwick, 2006; Adams and Cory, 2007). Another important function of Bcl-xL during cell death stimuli in cancer cells, however, is to increase the release of ATP through enhanced VDAC opening, to help the cell overcome stress and to decrease the probability of MOMP (Vander Heiden et al., 2001; Gottlieb et al., 2002). In one of the first studies of Bcl-2 family proteins in the synapse, it was found that injection of either Bcl-xL or ATP into the squid presynaptic terminal enhanced synaptic transmitter release to a similar

magnitude (Jonas et al., 2003). ATP injection also occluded the effect of Bcl-xL, suggesting that the two agents act via the same mechanism. The channel activity of mitochondria during synaptic transmission was also inhibited by small molecule Bcl-xL inhibitors (Hickman et al., 2008) suggesting that presynaptic plasticity may depend on ATP release from mitochondria regulated by the Bcl-2 family. In addition, these findings raised the intriguing question of whether Bcl-xL regulates not only the release but also the manufacture of ATP during synaptic transmission.

MITOCHONDRIAL METABOLIC PLASTICITY REGULATES SYNAPTIC PROPERTIES IN HIPPOCAMPAL NEURONS

Unlike in the high fidelity synapse of the squid where long term synaptic responses are relatively unvaried, in the mammalian hippocampus, long-term changes in synaptic transmission in both directions occur, and these changes may underlie learning and memory formation. In cultured hippocampal neurons, overexpression of Bcl-xL produces an increase in mitochondrial targeting to synaptic sites, and an enhancement in spontaneous and evoked synaptic responses (Li et al., 2008, 2013). Depletion of Bcl-xL produces the opposite findings. Furthermore, Bcl-xL overexpression is correlated with structural alterations in the synapse including enhanced expression of synaptic vesicle numbers, increased synaptic vesicle markers and an increase in postsynaptic markers, consistent with an increase in size and number of synapses. The Bcl-xL-dependent increase in localization of mitochondria to the synapse suggests a link between mitochondria and the marked alterations of synaptic structure found in developing and plastic synapses.

Bcl-xL INCREASES BIOENERGETIC EFFICIENCY BY CLOSING AN UNCOUPLING LEAK WITHIN THE ATP SYNTHASE

A link between synaptic alterations and metabolism produced by Bcl-xL was provided by studies on cultured hippocampal neurons overexpressing or depleted of Bcl-xL (Alavian et al., 2011; Chen et al., 2011). Overexpression of Bcl-xL in resting neurons led to a large (almost 100%) increase in cytoplasmic ATP levels. Surprisingly, this was accompanied by a decrease in neuronal oxygen uptake, as measured with oxygen-sensitive electrodes positioned over single neurons, and a decrease in aerobic glycolysis, consistent with the notion that Bcl-xL overexpression increases mitochondrial bioenergetic efficiency. Interestingly, Bcl-xL was found to markedly increase oxygen uptake during activity compared to controls, in keeping with previous findings of an increase in mitochondrial biomass and larger synapses (Li et al., 2008; Berman et al., 2009; Alavian et al., 2011). Despite this activity-dependent increase in oxygen uptake, however, calculations revealed that the fraction of total oxygen uptake used to make ATP during activity is much higher in Bcl-xL expressing neurons than controls, consistent with an increase in bioenergetic efficiency (Alavian et al., 2011; Chen et al., 2011). Bcl-xL depletion reversed the effects on metabolism, decreasing ATP production and increasing oxygen uptake by the resting cells.

How could these alterations in metabolic efficiency take place? As stated above, Bcl-xL increases the conductance of VDAC to release ATP from mitochondria in both cancer cells and squiid synapse. In addition to releasing ATP, however, in hippocampus Bcl-xL was found to interact directly with the ATP synthase to maximize the efficiency of production of ATP. Oxidative phosphorylation is the main source of ATP formation in neurons and requires coupling of electron transport (H+ pumping out of the mitochondrial matrix) to ADP phosphorylation [movement of H+ ions through the F_1F_0 ATP synthase (Complex-V)]. By measuring H+ ion movement using the pH sensitive dye ACMA in submitochondrial vesicles (SMVs) enriched in ATP synthase, it was demonstrated that a leak of H+ ions was always present in the SMVs, but that the leak was greatly attenuated by the addition of ATP or ADP in the presence of endogenous Bcl-xL. Using multiple biochemical and imaging approaches, the site of interaction of Bcl-xL within the F_1F_0 ATP synthase was localized to the beta subunit of the enzymatic portion (F_1) of the ATP synthase close to where ATP (ADP) normally binds (Alavian et al., 2011; Chen et al., 2011).

Closure of the leak within the ATP synthase in the presence of Bcl-xL produces mitochondrial plasticity in the form of an increase in metabolic efficiency; Bcl-xL aids actively firing neurons to produce increases neurotransmitter release (Jonas et al., 2003; Li et al., 2008, 2013), consistent with the possibility of a still-to-be-proven connection between the increase in metabolic efficiency and the long-term higher efficacy of synaptic transmission found in Bcl-xL expressing neurons.

In contrast, opening of the Bcl-xL-regulated leak results in metabolic inefficiency. This is confirmed in detail in neurons in which Bcl-xL had been genetically depleted. These neurons display a fluctuating mitochondrial membrane potential and a marked depolarization in the presence of the ATP synthase inhibitor oligomycin, underlining a necessity for Bcl-xL in metabolic efficiency and in interaction with the ATP synthase (Chen et al., 2011). The exact location of the leak, within the ATP synthase complex, is suggested by the next set of findings.

PERMEABILITY TRANSITION IS THE GREAT UNCOUPLER

The largest matrix-calcium-regulated uncoupling mechanism in mitochondrial inner membrane is PT. PT comprises a rapid increase in permeability of the mitochondrial inner membrane to solutes causing severe osmotic disarray. If it is not reversed, PT leads to structural breakdown of the mitochondrial matrix accompanied by outer mitochondrial membrane rupture and cell death. The interaction of this kind of mitochondrial cell death with apoptotic death produced by MOMP has been hotly debated. Although the two types of cell death may be overlapping, it is safe to say that PT is associated with necrotic cell death such as is found in ischemia or injury whereas MOMP may have a more important role in development and genetically predetermined death (Dejean et al., 2006; Bonora et al., 2014). Inter-membrane space pro-apoptotic factors such as cytochrome c and Smac/DIABLO are released during both forms of cell death. In MOMP, outer membrane permeabilization leads to release of these factors, whereas in PT, rupture of the outer membrane after inner membrane swelling leads to release of pro-apoptotic factors into the cytosol (Galluzzi et al., 2009). PT has been extensively studied for its role in ischemic injury in brain, heart and other organs as well as in neurodegenerative conditions (Bernardi, 2013; Bonora et al., 2014). PT is induced by calcium influx into

the matrix, ROS, inorganic phosphate and intracellular acidification (Szabo et al., 1992). It is inhibited by ATP/ADP and by Mg^{2+} (Kowaltowski et al., 1998; Crompton, 1999). The pharmacological agent most useful for inhibition of PT is cyclosporine A (CsA) which displaces the PT co-factor, cyclophilin D (CypD) from binding (Szabo and Zoratti, 1991; Giorgio et al., 2009). Recent reports have also confirmed the increased opening of PT by polyphosphates, chains of 10–100 s of repeating phosphates linked by ATP-like high energy bonds (Abramov et al., 2007; Seidlmayer et al., 2012) that have also been linked to causing plasma membrane excitability directly (Holmstrom et al., 2013; Stotz et al., 2014).

In recent years there has been much interest in identifying the molecules that form the mitochondrial PT pore (mPTP) and in understanding its regulation. A critical role for VDAC has been identified but VDAC appears to form the outer membrane passageway for a channel that must span both membranes (Baines et al., 2007). The ANT was put forth as a possible pore-forming molecule. Purified ANT forms active channels when reconstituted into proteoliposomes, and its physical interaction with VDAC, hexokinase, and CypD positioned it to be at the center of a supramolecular complex forming PT (Brustovetsky et al., 2002). However, genetic depletion of ANT does not abolish PT and mitochondria isolated from ANT knock out animals still retain the ability to undergo PT (Javadov et al., 2009). In contrast, mitochondria from CypD null mice are extremely resistant to CsA-inhibited, calcium-induced PT and to certain forms of ischemic cell death (Baines et al., 2005; Nakagawa et al., 2005). Although it is not suspected that CypD, which is soluble and not membrane imbedded, forms the PT pore, it is an important pore regulator of the core complex, by binding to the stator arm of the mitochondrial F1Fo ATP synthase, specifically to OSCP (Giorgio et al., 2009); this suggests that the PT pore may be contained within the ATP synthase complex. In support of this, it has been reported that ATP synthase dimers participate in mPTP formation (Giorgio et al., 2013). This idea is attractive because isolated purified ATP synthasomes (or SMVs) contain all the inner membrane PT regulatory components including the phosphate carrier, ANT and CypD as well as most of the proteins found within the ATP synthase complex (Ko et al., 2003; Chen et al., 2004). In addition, subunit c was found to participate in mPTP function within the complex composed of the ATP synthase, ANT, and PiC (Azarashvili et al., 2014). The authors of this report hypothesized that dephosphorylation of subunit c occurs in the presence of high matrix calcium, leading to opening of mPTP and mitochondrial swelling in a CsA-sensitive fashion. Subunit c of F0F1-ATPase might therefore act as a structural or regulatory component of the mPTP complex in a phosphorylation-dependent manner.

THE c-SUBUNIT OF THE F_1F_0 ATP SYNTHASE FORMS THE mPTP

Any leak of the ATP synthase complex must be present in the membrane-embedded portion (F_O). The F_O is connected to the enzymatic portion (F_1) by a central stalk and a peripheral arm called the stator. This arm bends over the catalytic subunit. Protons pumped out of the matrix by the electron transport complexes re-enter the matrix by traveling down their electrochemical gradient through a translocator at the junction between the outer wall of subunit-c and subunit-a of the F_O. The energy dissipated by H+ translocation creates a conformational change in the alpha and beta subunits resulting in the synthesis of ATP from ADP and Pi (Watt et al., 2010). When viewed from the inter-membrane space, the denuded octameric c-subunit appears as a ring with a central pore-like structure that is normally obscured by the F_1 stalk components gamma, delta and epsilon (Pogoryelov et al., 2007). The c-subunit ring could therefore form an ion conducting channel that would allow for uncoupling if the stalk partially or completely dissociated from it. Older studies suggested that the c-subunit of F_O has pore-forming capability (McGeoch and Guidotti, 1997; Pavlov et al., 2005), and a recent study has found that depletion of all three isoforms of c-subunit by siRNA in cells leads to protection from the onset of PT in response to calcium or oxidant challenge (Bonora et al., 2013). In addition, over-expression of the c-subunit under certain conditions can pre-dispose to PT (Bonora et al., 2013).

To test the hypothesis that the c-subunit ring forms the pore of the mPTP, we analyzed the ability of the purified c-subunit to form a pore (Alavian et al., 2014). Indeed, recordings of the purified c-subunit yield a multi-conductance, voltage dependent channel. Reconstructing regulation of the c-subunit pore by recording from increasingly purified groups of inner membrane proteins demonstrate that there are layers of regulation of the c-subunit leak channel. In mitochondria or inner membrane preparations (lacking the outer membrane) calcium activates the c-subunit leak channel while CsA and ATP/ADP inhibit it. Removal of the F_1 by urea treatment of the inner membrane or removal of CypD by purification of ATP synthase monomers causes the c-subunit channel to lose sensitivity to CsA and calcium but not to ATP/ADP, suggesting that the CsA/calcium binding site is associated with the F_1 portion but that a second ATP binding site exists in the F_O. This is consistent with reports identifying the CsA/CypD binding site on OSCP (Giorgio et al., 2009, 2013). Channel activity of the purified c-subunit is inhibited by the purified beta subunit of F_1 and by ATP/ADP, suggesting a structural rearrangement whereby the stalk of the ATP synthase inhibits opening of the c-subunit leak channel, aided by ADP/ATP binding to the beta subunit or by the pharmacological agent CsA binding to OSCP (**Figure 2**).

Interestingly, exposure of mitochondria to high calcium unmasks the F_O by separating the F_1 from the F_O, indicating that calcium activates the PT pore by facilitating this separation. The unmasking of the c-subunit leak channel is prevented by pre-exposing mitochondria to CsA or ADP. In addition, the unmasking of the c-subunit in response to calcium is greatly attenuated in mitochondria isolated from the CypD null mouse (Baines et al., 2005; Nakagawa et al., 2005) that is resistant to calcium induced PT. ATP synthase channel activity is also enhanced by the addition of CypD protein to purified ATP synthase monomers, implicating CypD as a regulator of the c-subunit leak channel, opposed by CsA and ADP (Alavian et al., 2014). Therefore, the leak channel, which has a higher probability of closed than open state in intact and enzymatically active synthase complexes, opens in the presence of CypD and mitochondrial calcium influx by dissociation of

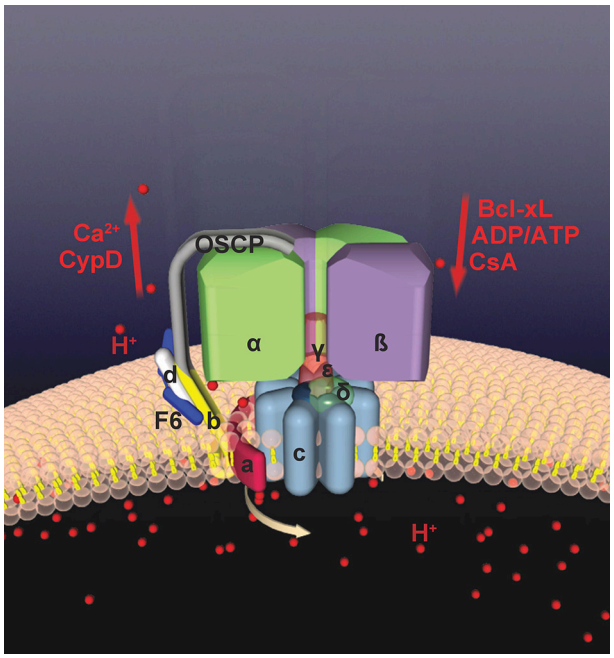

FIGURE 2 | Bcl-xL binds to ATP synthase and regulates mPTP. Shown are binding locations between various regulatory molecules and the ATP synthase. The ATP synthase has recently been found to form the mPTP. The c-subunit forms a leak within the ATP synthase which is closed by F1 interaction. Bcl-xL and ATP/ADP close mPTP by interacting with the beta subunit of the F1. CsA and CypD bind to OSCP; CypD enhances opening of mPTP by releasing the F1 from the c-subunit pore, opposed by the actions of CsA.

the OSCP-bound catalytic subunits. Exposure of the membrane (F_O) portion and inner mitochondrial membrane depolarization could, therefore, be reversible through an OSCP (or beta subunit)-dependent conformational modification of the complex (**Figure 2**).

mPTP OPENING CORRELATES WITH CELL DEATH IN ACUTE ISCHEMIA, ROS DAMAGE OR GLUTAMATE EXCITOTOXICITY

PT occurs acutely during such events as ischemic excitotoxicity or high ROS production that can occur during rapid onset of brain or cardiac ischemia (Baines, 2009). Our studies and those of others (Bonora et al., 2013) show that excitotoxic and ROS-induced cell death are greatly attenuated upon depletion of the c-subunit by shRNA in neurons and other cells (Bonora et al., 2013; Alavian et al., 2014); however, cell death protection by c-subunit depletion is not further attenuated by CsA, suggesting that the c-subunit forms the inner mitochondrial membrane target of the CsA-sensitive complex (Alavian et al., 2014). Opening of the PT pore under stress may also be sensitive to Bcl-xL binding to the F_1 beta subunit, as suggested by our recent studies (Chen et al., 2011; Alavian et al., 2014). In contrast to c-subunit depletion, over-expression of the wild type c-subunit (Bonora et al., 2013) or mutation of the c-subunit to form a high-conductance leaky c-subunit (Alavian et al., 2014) predisposes to enhanced cell death upon excitotoxic or ROS stimulation. Death under these conditions is not sensitive to CsA, presumably because the leaky pore prevents normal regulation by components of F1. In summary, our data and other recent studies suggest that the c-subunit of the ATP synthase forms the pore of a large complex of regulatory proteins within the ATP synthase that comprises the mPTP. Upon calcium or ROS exposure, opening of the pore by relative removal of the F_1 from the membrane produces mitochondrial membrane depolarization, abrogation of the H+ gradient across the inner membrane, and cell death.

PRO-APOPTOTIC PROTEOLYTIC CLEAVAGE FRAGMENT OF Bcl-xL CAUSES LARGE CONDUCTANCE MITOCHONDRIAL ION CHANNEL ACTIVITY CORRELATED WITH HYPOXIC SYNAPTIC FAILURE

In the synapse, ROS or excitotoxicity may not produce death of the soma, but in contrast severe neuronal stress may result in long lasting synaptic depression or a decline in neuronal excitability. A decline in neurotransmitter release or recycling may subsequently mark a synapse for elimination, followed by somatic death if many synapses are sequentially eliminated. Before the onset of decline, a set of changes occurs in mitochondrial membrane activity that negatively affects synaptic function.

Under pro-apoptotic conditions in growth-factor deprived cancer cell lines, Bcl-2 family proteins activate large mitochondrial outer membrane channel activity that participates in release of pro-apoptotic factors from mitochondria (Antonsson et al., 2000; Dejean et al., 2005), either in the absence of any change to the properties of the inner membrane or, as may occur during ischemia, accompanying induction of calcium or ROS-induced depolarization and loss of osmotic regulation (permeability transition) of the inner mitochondrial membrane (Tornero et al., 2011; D'Orsi et al., 2012; Perez-Pinzon et al., 2012). In the synapse, the effects of hypoxia serve as a model to study the role of Bcl-xL in mitochondrial ion channel events activated during severe neuronal injury (Jonas et al., 2004, 2005). The presynaptic terminal is very sensitive to hypoxia, which attenuates synaptic transmission over 10–30 min. Patch clamp recordings of mitochondrial membranes at rest during hypoxia reveal large conductance activity not found frequently in controls. The channel activity is larger than that induced by pipette-mediated application of recombinant full length Bcl-xL protein and is mimicked by activity of recombinant proteolytically-altered Bcl-xL (ΔN Bcl-xL) that forms a large conductance (pro-apoptotic or MOMP-like) channel activity in the outer mitochondrial membrane. In contrast, large conductance activity of mitochondria during hypoxia is prevented by pre-treatment of the synapse with a pan-caspase/calpain inhibitor that prevents the cleavage of Bcl-xL. Appearance of the channel associated with ΔN Bcl-xL during hypoxia most likely arises from specific proteolysis of Bcl-xL and not from general injury, because levels of VDAC are preserved in both caspase/calpain inhibitor-treated and untreated hypoxic synapses.

In contrast to the response to injection into the synapse of full length Bcl-xL, which causes synaptic potentiation, injection of ΔN Bcl-xL protein produces a marked synaptic depression (Jonas et al., 2003; Hickman et al., 2008). The time course of run-down of synaptic responses matches that of hypoxia, suggesting a correlation between the two types of synaptic decline.

More evidence that Bcl-xL protein can produce two different conductance level channel activities came from *in vivo* studies with the Bcl-xL inhibitor ABT-737. When applied to mitochondria within the squid presynaptic terminal just before healthy synaptic transmission, ABT-737 inhibited the channel activity of mitochondrial membranes induced by synaptic stimulation, suggesting that full length Bcl-xL is necessary for this activity (Hickman et al., 2008). ABT-737, however, also attenuated the channel activity of ΔN Bcl-xL (Hickman et al., 2008). ABT-737 reversed the decline in synaptic responses produced by hypoxia or by direct injection of ΔN Bcl-xL into the terminal and enhanced synaptic function, suggesting that the amplitude of activity at mitochondrial outer membranes may determine the direction of changes in synaptic strength. In addition, by attenuating the channel activity of ΔN Bcl-xL in an *in vivo* model of transient global ischemia, ABT-737 effectively prevented delayed cell death of hippocampal CA1 neurons (Ofengeim et al., 2012). Ischemic death of CA1 neurons was also prevented in a KI mouse containing a form of Bcl-xL resistant to caspase/calpain cleavage (Ofengeim et al., 2012) confirming the specific role of ΔN Bcl-xL in the onset of cell death in hippocampal CA1 neurons during global ischemic brain injury.

SYNAPTIC RESPONSES DECLINE DURING LONG TERM DEPRESSION IN ASSOCIATION WITH Bcl-xL-REGULATED Bax-INDUCED MITOCHONDRIAL CHANNEL ACTIVITY

The enhancement of synaptic responses by full length Bcl-xL could be related to a difference not only in size of the conductance of its mitochondrial ion channel compared to that of pro-apoptotic molecules such as Bax or ΔN Bcl-xL, but also to a difference in function of the activity. A key characteristic of the ion channel activity of Bcl-xL is that it can induce ATP exchange across mitochondrial membranes (Vander Heiden et al., 2000, 2001) while activity of ΔN Bcl-xL or Bax causes release of pro-apoptotic factors such as cytochrome c and in addition causes caspase activation. The delicate balance between pro- and anti-apoptotic Bcl-2-related activities may thereby regulate mitochondrial metabolism at times of stress and may control the timing of eventual synaptic rundown or death of the soma if neuronal stress overwhelms anti-apoptotic capabilities (Plas and Thompson, 2002). Release of factors such as cytochrome c not only activates downstream caspases that inactivate cellular processes, but also directly compromises function by depriving mitochondria of electron transport carriers; These features may all contribute to the decline in synaptic responses found after full length Bcl-xL cleavage or Bax activation and formation of large conductance outer membrane channel activity.

LTD REPRESENTS NORMAL SYNAPTIC PLASTICITY BUT CAN BE A MARKER OF SYNAPTIC DEGENERATION

Long term synaptic depression (LTD) caused by low frequency stimulation or by cell signaling is a normal mechanism of synaptic plasticity opposite in some ways to long term potentiation (LTP) brought on by high frequency stimulation (Malenka and Bear, 2004). Despite its role in normal synaptic plasticity, however, long term depression can also serve as a marker for a pre-degenerative synaptic state. In hippocampal CA3 to CA1 synapse, low synaptic activity leads to a long lasting decline in synaptic efficacy, brought about in part by removal of postsynaptic receptors (Malinow and Malenka, 2002; Kessels and Malinow, 2009); this state can be quite stable and may never lead to somatic demise. It has been described recently that mitochondria are important for a form of LTD associated with normal synaptic plasticity in hippocampal CA1 neurons. In the CA1 dendrite, low frequency activity causes Bcl-xL-sensitive mitochondrially-mediated release of cytochrome c followed by low level activation of caspase 3, which leads to the removal of postsynaptic glutamate receptors from the plasma membrane, resulting in a form of LTD (Li et al., 2010). In addition to these findings, however, degenerative changes may also be associated with LTD. In synapses from $Bax^{-/-}$ mice treated with the toxic Abeta protein, LTD was prevented, implying that Bax actions at mitochondria are necessary for this form of degenerative hippocampal LTD (Olsen and Sheng, 2012; Sheng, 2014). In a model of developmental axonal targeting in spinal neurons, both mitochondrial Bax and caspase 6 activation were found to control axonal loss in response to nerve growth factor withdrawal (Nikolaev et al., 2009). In this scenario, the N-terminus of amyloid precursor protein (APP) bound to death receptor 6 (DR6) to initiate an intracellular cascade resulting in mitochondrial-dependent axonal demise.

The difference between decreased activity and damage was made more clear in a recent study of neurite growth arrest in cultured neurons depleted of Bcl-xL by siRNA. Declining Bcl-xL levels prevented normal outgrowth and branching of neuronal processes over 4 weeks in culture before any somatic death occurred (Park et al., 2014). In contrast to the slowly occurring growth arrest found upon Bcl-xL depletion, loss of neurites takes place with a much more rapid timescale after a brief death stimulus of hypoxia. This hypoxia-induced loss is greatly attenuated by normal endogenous Bcl-xL levels and by depletion of DR6, which prevented neurite disruption especially in cells previously depleted of Bcl-xL. These findings raise the interesting question of whether the upregulation of DR6 and neurite growth arrest in Bcl-xL-depleted neurons are related to metabolic changes in dendritic and axonal mitochondria such as would naturally occur in underused synapses.

In support of this notion, Bcl-xL increases the number of synapses housing mitochondria through its actions on the mitochondrial fission protein Drp1 (Li et al., 2008, 2013; Berman et al., 2009). Synapses containing mitochondria have larger synaptic vesicle clusters by light and electron microscopy, suggesting that mitochondria are crucial for this form of synaptic plasticity. Recent studies suggest that axonal branching may also be regulated by mitochondrial positioning: The MARK and SAD family member kinase NUAK1 helps capture mitochondria at axonal branch points (Courchet et al., 2013; Lewis et al., 2013), possibly related to increased activity at these sites. Mitochondria are known to pause at sites where activity is high and they continue to move past sites where activity is low, regulated by specific docking proteins, kinases, and external growth factor cues (Chada and Hollenbeck, 2003; Saxton and Hollenbeck, 2012; Vaccaro and Kittler, 2013); these processes may contribute to synaptic plasticity (Obashi and Okabe, 2013; Sun et al., 2013). The sites where mitochondria dock in preparation for axon or dendritic

branching are sites that may form hot spots for mRNA accumulation, protein translation, and vesicular trafficking, as receptors for neurotransmitters and growth factors are inserted or removed to account for the new functions of growing neuritic processes. These studies emphasize that that cues from the extracellular environment during neuronal development or neuronal plasticity may regulate mitochondrial positioning and metabolism as well as protein translation and vesicular trafficking to determine the eventual outcome of neuronal network formation.

CONCLUSIONS

We have focused on Bcl-xL as an example of a survival protein that has more extensive roles than prevention of cell death. In order to promote neuronal survival, perhaps Bcl-xL responds to neuronal activity by translocating to mitochondrial enhancing the efficiency of metabolism by regulating ATP production synthesis via changes in calcium uptake and release and the efficiency of ATP synthase activity. Thus, under normal circumstances, Bcl-xL coordinates mitochondrial activity with enhanced neurotransmitter vesicle recycling. In the opposite scenario, Bcl-xL protein loss from mitochondria and intracellular organelles may mimic synaptic disuse leading to depression of synaptic responses, pro-apoptotic mitochondrial conductance changes, metabolic compromise and eventual loss of neurite outgrowth. This type of synaptic rundown is also found acutely in hypoxic brain damage, synaptic injury, and in long-term neurodegenerative changes.

At synaptic mitochondria, pro-apoptotic mitochondrial conductance changes regulated by Bcl-xL include not only large conductance activity of the outer mitochondrial membrane, but also changes at the inner membrane characterized by the opening of a large leak conductance found within the ATP synthase c-subunit. We have evidence that this highly regulated leak decreases the efficiency of ATP generation and may lead to acute mitochondrial depolarization and osmotic disarray characteristic of mPT. Bcl-xL, ATP/ADP and certain pharmacological agents deter the opening of this leak, rescuing neurons from necrotic and some forms of apoptotic death. On the other hand, increased probability of leak closure within the c-subunit of ATP synthase may lead to long-term enhancement in the efficacy of synaptic responses and perhaps neuroprotection.

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Metabolism leaves its mark on the powerhouse: recent progress in post-translational modifications of lysine in mitochondria

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Lysine modifications have been studied extensively in the nucleus, where they play pivotal roles in gene regulation and constitute one of the pillars of epigenetics. In the cytoplasm, they are critical to proteostasis. However, in the last decade we have also witnessed the emergence of mitochondria as a prime locus for post-translational modification (PTM) of lysine thanks, in large measure, to evolving proteomic techniques. Here, we review recent work on evolving set of PTM that arise from the direct reaction of lysine residues with energized metabolic thioester-coenzyme A intermediates, including acetylation, succinylation, malonylation, and glutarylation. We highlight the evolutionary conservation, kinetics, stoichiometry, and cross-talk between members of this emerging family of PTMs. We examine the impact on target protein function and regulation by mitochondrial sirtuins. Finally, we spotlight work in the heart and cardiac mitochondria, and consider the roles acetylation and other newly-found modifications may play in heart disease.

Keywords: acetylation, succinylation, malonylation, glutarylation, heart, sirtuin, Sirt3, Sirt5

INTRODUCTION

Though mitochondria are the primary site for ATP production, they have also emerged as hubs of control within a cell. What fuel source to use, whether to divide or fuse, to live or die—these are only a few of the decisions made at the level of mitochondria that require the integration of signals about nutrient availability, redox status, and overall health of the cell. Defining these signals is key to understanding mitochondrial function and designing therapies to minimize mitochondrial dysfunction during disease. Evidence indicates that mitochondrial processes are subject to modulation by a number of post-translational modifications (PTM), including phosphorylation of serines, threonines, and tyrosines, as well as through the redox-induced modification of reactive cysteines to yield glutathionylated and S-nitrosylated proteins (reviewed in Foster et al., 2009; O'Rourke et al., 2011; Mailloux et al., 2014).

In this review, we consider the PTM of lysine residues, concentrating primarily on those found in the mitochondrion. What has become clear over the last few years is that there is an emerging class of lysine modifications that stem from the reaction of proteins with thioester-CoenzymeA (CoA) byproducts of metabolism. We begin by surveying the field of mitochondrial lysine acetylation, before introducing more recently discovered PTMs, including succinylation, malonylation, and glutarylation. In each case, we address some key questions. What protein substrates are modified and how is function affected? Are these PTMs modulated by enzymes and, if so, what factors govern their activity? How dynamic are these processes and what is their stoichiometry? The answers to these questions will help shape our perception of these new PTMs, i.e., do they constitute a novel form of signaling, or is their accumulation simply a form of "metabolic stress" to be minimized? Finally, where applicable, we spotlight recent work in cardiac mitochondria, and examine the role of these PTMs in models of heart disease.

LYSINE ACETYLATION

Biological acetylation of histones was first reported by Allfrey et al. (1964), though initial measurements primarily captured N-terminal acetylation, i.e., the reaction with the α-amino group of the mature histone's first amino acid. It was not until 1968 that investigators realized there was a second, more labile, population of acetylated residues in histones, labeled at the ε-amino group of internal lysine residues (Gershey et al., 1968). The transfer of an acetyl group from acetyl-CoA to the ε-amino group neutralizes lysine's positive charge. In the nucleus, this is a critical step in gene regulation, and is acutely regulated by the specific action of enzymes: the histone acetyl transferases (HATs) and deacetylases (HDACs). In the mid-2000's, advances in mass spectrometry coupled with the development of acetylation specific antibodies greatly expanded the catalog of lysine acetylated proteins beyond the nucleus, and mitochondrial proteins figure prominently. Lysine acetylomes have now been compiled from bacteria and yeast to fruit flies (Zhang et al., 2009; Weinert et al., 2011; Henriksen et al., 2012) and mammals. Given the prokaryotic ancestry of mitochondria, we first consider evidence for acetylation in bacteria.

PROTEIN LYSINE ACETYLATION IN PROKARYOTES

In prokaryotes, acetylation has emerged as a central regulatory mechanism that coordinates metabolism in response to changes in nutrient status (Wang et al., 2010). In *S.enterica,* 191 proteins are reported to be lysine-acetylated and in *E.coli* this number currently reaches 349 (Wang et al., 2010; Zhang et al., 2013). Metabolic enzymes (e.g., aldolase, Pdh, and Mdh, to name a few) are common substrates for acetylation and ontologic classification shows that acetylation on metabolic regulators is more frequent than that observed, for example, on ribosomal proteins. The prokaryotic enzymes, Pat and CobB, regulate bacterial protein lysine acetylation and deacetylation respectively (Starai et al., 2002; Starai and Escalante-Semerena, 2004; Wang et al., 2010). The balance between acetylation/deacetylation is influenced by several conditions, including switching carbon sources (e.g., from glucose to citrate), growth phase, and availability of acetylphosphate (AcP) (Yu et al., 2008; Zhang et al., 2009; Wang et al., 2010; Weinert et al., 2013a). Consensus sites that may serve as hotspots for lysine acetylation/deacetylation have been examined and, although no distinct patterns emerge, having tyrosine or histidine at site +1 frequently correlates with acetylation (Zhang et al., 2009), though not in all bacterial species examined (Kim et al., 2013). Estimates of stoichiometry in *E.coli*, indicate that, for the bulk of acetylated lysines, occupancy is below 1% (Weinert et al., 2013a). This is consistent with more recent calculations, showing that the stoichiometry for 82% of acetylated sites was equal or below 10% and only a small fraction of sites (4%) exhibited stoichiometries larger than 20% (Baeza et al., 2014). High acetylation stoichiometries were found in proteins participating in metabolic pathways including the pentose phosphate pathway, glycolysis and the tricarboxylic acid (TCA) cycle (Baeza et al., 2014).

The observation that acetylation occupancy is low for the majority of sites prompts the question as to whether it serves as a regulatory PTM or is simply a non-specific epiphenomenon of metabolism. Ultimately, the answer is likely a bit of both, though it would be prudent not to summarily dismiss a site's regulatory potential on the basis of low stoichiometry alone. Though most would agree that high site stoichiometry is a promising sign of its potential for protein regulation, it is no guarantee. Conversely, low PTM stoichiometry can have profound regulatory consequences if the target performs a key step in feed-forward processes such as enzymatic cascades or co-operative systems.

Regardless of stoichiometry, there are clearly examples of functional regulation by acetylation/deacetylation in prokaryotes. In *S.enterica* acetylation of GapA (prokaryotic homolog of Gapdh) stimulates glycolysis, while deacetylation reverses the equilibrium toward gluconeogenesis (Wang et al., 2010). This model illustrates one example where acetylation promotes the enzymatic activity of its substrate and feeds forward for the production of more acetyl-CoA through glycolysis. An example of feedback inhibition in *S.enterica* is the regulation of Acs (acetyl-CoA synthetase) by Pat and CobB. When acetyl-CoA is in excess, Acs is acetylated and deactivated by Pat and when glycolytic substrates become limited, CobB deacetylates Acs and activates the enzyme to synthesize acetyl-CoA from acetate (Thao and Escalante-Semerena, 2011). Another example in *E.coli* is the regulation of RNA polymerase (RNAPα) via acetylation at the C terminal domain (CTD) (Lima et al., 2012). With bacterial acetylomes expanding, we expect that more examples of protein regulation by acetylation will emerge thus improving our understanding of the biological significance of this modification.

PROTEIN LYSINE ACETYLATION IN YEAST AND FLIES

Acetylomes have been reported for model organisms such as *Saccharomyces cerevisiae* (Brewer's yeast) and *Drosophila melanogaster* (fruit fly), while for others, such as *Caenorhabditis elegans* (nematode worm) and *Danio rerio* (zebrafish), broad-scale proteomic assessments of lysine acetylation have yet to be described. Current studies have identified 959 or 1059 acetylated proteins in yeast (Henriksen et al., 2012; Weinert et al., 2013b) and 1013 acetylated proteins in the SL2 fruit fly cell line (Weinert et al., 2011). Proteins from every functional class are subject to acetylation (e.g., RNA processing, protein synthesis) while almost every enzyme participating in major metabolic pathways (e.g., glycolysis, gluconeogenesis) is acetylated in yeast (Henriksen et al., 2012). The evolutionary conservation of acetylated lysines is higher than non-acetylated lysines (Weinert et al., 2011; Henriksen et al., 2012) and sites of lysine acetylation are more tightly conserved than sites of phosphorylation (Weinert et al., 2011). Nevertheless, the stoichiometry of phosphorylation in yeast is significantly higher than acetylation, where the majority of acetylated sites have stoichiometries lower than 1% (Weinert et al., 2013b). The few exceptions with high acetylation stoichiometries are proteins already known to undergo acetylation by specific acetyl-transferases (largely related to acetylation in the nucleus). For other proteins, acetylation may occur as a low-frequency, non-enzymatic reaction driven by the concentration of acetyl-CoA (Weinert et al., 2013b). As discussed below, non-enzymatic modification is one of the proposed mechanisms underlying the acetylation of proteins in mitochondria (Newman et al., 2012; Wagner and Payne, 2013; Weinert et al., 2013b).

CHEMICAL AND ENZYMATIC CONTROL OF MITOCHONDRIAL ACETYLATION/DEACETYLATION IN MAMMALS

As in prokaryotes, lysine acetylation in mitochondria is tightly coupled to nutrient availability. The mechanism underlying acetylation, however, is still debated. It has been recently suggested that elevated *p*H in the mitochondrial matrix is favorable for the acetylation reaction (Wagner and Payne, 2013). Moreover, the mitochondrial matrix is where enzymes of the TCA cycle maintain high concentrations of acetyl-CoA. Together, these conditions would favor non-enzymatic formation of acetyl-lysine (Wagner and Payne, 2013). Additionally, Gcn5l1 is recently identified as a putative mitochondrial acetyltransferase in HepG2 cells (Scott et al., 2012). Gcn5l1, shares sequence similarity with bacterial acetyltransferases, localizes to mitochondria (both in the matrix and the IMS), and promotes mitochondrial protein acetylation in cells and in cell-free systems (Scott et al., 2012). Substrates of Gcn5l1 include electron transport chain (ETC) subunits Ndufa9 and Atp5a. It should be noted however that for full activity, Gcn5l1 requires additional mitochondrial factors and/or partners that are presently unknown. An intriguing observation in the two studies examining non-enzymatic acetylation or

Gcn5l1-mediated acetylation is that mitochondrial proteins are more prone to acetylation when in their denatured state (Scott et al., 2012; Wagner and Payne, 2013).

By contrast to acetylation, there is no dearth of information regarding enzyme-driven mitochondrial deacetylation. Mammals host a family of seven genes called sirtuins 1 through 7 (Sirt1-7), due to their sequence homology to yeast silent information regulator 2 (Sir2p) (Haigis and Guarente, 2006). Three of the sirtuins (Sirt3, Sirt4 and Sirt5) are found in mitochondria (Onyango et al., 2002; Schwer et al., 2002; Michishita et al., 2005; Shi et al., 2005). The sirtuins use NAD^+ co-substrate, which makes them particularly sensitive to fluctuations in metabolism. Notwithstanding their homology to yeast Sir2p, not all of the mitochondrial isoforms are equally effective as NAD-dependent deacetylases. Of the three mitochondrial sirtuins, Sirt3 appears to have the broadest deacetylase activity *in vivo* and will be discussed in subsequent sections (Lombard et al., 2007). Sirt4 is more widely recognized for its ADP-ribosyl-transferase activity (Haigis et al., 2006; Ahuja et al., 2007), although some suggest this may not be its major enzymatic role (Du et al., 2009). Sirt4 *does* exhibit some deacetylase activity *in vitro* (Rauh et al., 2013) and may have a restricted, but important, set of substrates *in vivo.* Recently, Sirt4 has been implicated in the coordination of fatty acid metabolism (Laurent et al., 2013a,b), modulation of ATP release from mitochondria (Ho et al., 2013) and tumor suppression by inhibiting glutamine metabolism (Csibi et al., 2013; Jeong et al., 2013). It is likely that as our view of the PTM landscape in mitochondria expands, additional substrates and/or activities of Sirt4 will be uncovered. This is exemplified by the case of Sirt5, a sirtuin with limited deacetylase activity, whose function has become clearer since the identification of malonylation and succinylation (more later).

HARNESSING PROTEOMICS TO MAP PATHWAYS TARGETED BY ACETYLATION

The breadth of lysine acetylation on mitochondrial proteins was appreciated from the early proteome-wide studies that used cells such as HeLa, Jurkat, NIH/3T3, MV4-11 (an AML cell line) and A549 (an epithelial cell line) (Kim et al., 2006; Choudhary et al., 2009) and also mitochondria fractionated from mouse or human liver (Kim et al., 2006; Zhao et al., 2010). These studies are largely responsible for establishing the proteomic methodology used to identify acetylation sites. The workflow entails the enrichment of post-translationally modified peptides, first by precipitation of proteins (extracted from mitochondria, cells, or whole tissues), digestion with trypsin (and sometimes Lys-C endopeptidase from *Lysobacter enzymogenes*) and immunopurification of acetyl-lysine-bearing peptides using specific antibodies. Peptide capture is followed by chromatographic separation using high performance liquid chromatography and tandem mass spectrometry (MS/MS). The outcomes of such studies include the reporting of 133 acetylated proteins in mouse liver mitochondria (MLM) in 2006, 1395 acetylated proteins in Jurkat cells in 2009 and 1047 acetylated proteins in human liver in 2010 (Kim et al., 2006; Choudhary et al., 2009; Zhao et al., 2010). These methods have now been broadly applied and have proven integral to elucidating which biochemical pathways are subject to modulation by a variety of stimuli.

A central theme has been to examine precisely how lysine acetylation varies with nutrient availability and dietary composition. Examples include (i) fasting for 12 or 24 h (Kim et al., 2006; Hirschey et al., 2010; Rardin et al., 2013a), (ii) fasting and re-feeding (16 and 2 h respectively) (Still et al., 2013), (iii) a calorie restriction (CR) regimen (e.g., from 86.4 to 64.8 kcal/week for 2 or 3 months) (Hallows et al., 2011; Hebert et al., 2013), and (iv) high fat feeding (for 1, 7, or 13 weeks) (Hirschey et al., 2011). Changing the nutrition status elicits profound changes to the acetylation profile, but the outcome is often complex. For example, both high fat feeding and CR are reported to increase overall acetylation in liver mitochondria (Schwer et al., 2009; Hirschey et al., 2011; Hebert et al., 2013). A recent study using $Sirt3^{-/-}$ mice identified 1757 acetylated sites in liver mitochondria as targets for deacetylation by Sirt3 in the context of CR (Hebert et al., 2013). Another study using the same mice identified 283 acetylated sites as targets for deacetylation by Sirt3 during a period of 24 h of fasting (Rardin et al., 2013a).

Global scale lysine acetylomes have also helped map which biochemical pathways are affected. Besides mouse liver, acetylomes are also reported for 16 unfractionated rat tissues including heart (1294 acetylated proteins), brain (1653 acetylated proteins) and brown adipose tissue (705 acetylated proteins) and also human skeletal muscle (941 acetylated proteins) (Lundby et al., 2012). Comparisons across tissues revealed that acetylation occurs on proteins known to be physiologically important for each tissue. For example, in heart and skeletal muscle, 80% of the proteins involved in muscle contraction were acetylated. Moreover, owing to their high content in mitochondria, brown fat, skeletal muscle and heart exhibited acetylation on the highly abundant proteins of the TCA cycle and the ETC (Lundby et al., 2012). In the liver, a tissue with a very versatile metabolism, acetylation is prominent on proteins involved in: (i) glycolysis/gluconeogenesis, (ii) fatty acid oxidation, (iii) the TCA cycle, (iv) the ETC and ATP synthase, (v) the catabolism of branched chain amino acids and the urea cycle, (vi) ketogenesis and even the malate shunt, peroxisomal FAO and acetate metabolism. Examples of acetylated enzymes are summarized in **Table 1**. This table (based mainly on studies in liver) illustrates some of the recent trends in the field, including efforts to identify regulatory sites and sites that are *bona fide* targets for deacetylation by Sirt3. Indeed, studies with knockout mice reveal the importance of Sirt3 activity in maintaining many lysines in their deacetylated state. Nevertheless, there remains a considerable fraction of lysines whose acetylation status varies independently of Sirt3 genotype, while other acetylation sites remain static (Hebert et al., 2013; Still et al., 2013).

Amid the flood of data, considerable effort is now aimed at assessing the functional consequences of target protein acetylation. Usually the candidate lysine (K) is mutated to either glutamine (Q) to mimic the charge-neutralizing effect of adding an acetyl group, or arginine (R), to render the site unacetylatable. These approaches have been used, for example, to show that acetylation of four lysine residues on cytoplasmic malate dehydrogenase (Mdh1) stimulate its enzymatic activity (Zhao et al., 2010). By contrast, acetylation of long chain acyl-CoA dehydrogenase (Lcad) at K42 is inhibitory (Hirschey et al., 2010), as

Table 1 | Metabolic pathways and examples of acetylated enzymes/proteins identified by proteome-wide studies.

Metabolic pathway	Enzyme	Extended enzyme name	K-Ac site verified with functional assay	References	K-Ac site candidate for deacetylation by Sirt3	References
Glycolysis	ALDO	Aldolase	K147 (r) ↓	Lundby et al., 2012		
	GPD1	Glycerophosphate dehydrogenase 1	K120 (r) ↓	Lundby et al., 2012		
Gluconeogenesis	PEPCK1	Phosphoenolpyruvate carboxy kinase 1	K70, K71, K594 (h) ↓	Zhao et al., 2010		
Fatty acid oxidation	CPT2	Carnitine palmitoyl transferase 2			K510	Rardin et al., 2013a
	VLCAD	Very long chain Acyl-coA dehydrogenase			K299	Hebert et al., 2013
	LCAD	Long chain Acyl-CoA dehydrogenase	K42, K318, K322 ↓	Hirschey et al., 2010; Bharathi et al., 2013	K81, K322	Rardin et al., 2013a
	ACADSB	Short/Branched chain Acyl-CoA dehydrogenase			K284	Hallows et al., 2011
	SCHAD	Short chain L3-hydroxy-Acyl-CoA dehydrogenase			K202	Hallows et al., 2011
	EHHADH	Enoyl-CoA Hydratase/Hydroxyacyl-CoA dehydrogenase	K171, K346 (h) ↑	Zhao et al., 2010		
	ACAA2	AcetylCoA acyltransferase 2			K211	Rardin et al., 2013a
Tricarboxylic acid (TCA) cycle	CS	Citrate synthase			K49, K52	Rardin et al., 2013a; Hebert et al., 2013
	SDHA	Succinate dehydrogenase subunit A			K179	Hebert et al., 2013; Rardin et al., 2013a; Sol et al., 2012
	IDH2	Isocitrate dehydrogenase 2	K413 ↓	Yu et al., 2012	K360	Rardin et al., 2013a
	ACO2	Aconitase 2			K144	Hebert et al., 2013
	MDH2	Malate dehydrogenase 2	K239 ↓	Hebert et al., 2013	K239	Hebert et al., 2013; Rardin et al., 2013a
Electron transport chain/ ATP synthase	NDUFB11	Nadh-ubiquinone oxidoreductase subunit 11			K43	Hebert et al., 2013
	NDUFV3	NADH-Ubiquinone oxidoreductase flavoprotein subunit 3			K46	Hebert et al., 2013
	COX4	Cytochrome C oxidase subunit 4			K29, K60	Hebert et al., 2013; Sol et al., 2012
	ATP5O	ATP synthetase subunit 5O			K84, K97	Hebert et al., 2013; Rardin et al., 2013a
Aminoacid catabolism	GDH1	Glutamate dehydrogenase 1			K477, K480	Rardin et al., 2013a
	BCKDHA	Branched chain alpha ketoacid dehydrogenase A			K199	Hebert et al., 2013

(Continued)

Table 1 | Continued

Metabolic pathway	Enzyme	Extended enzyme name	K-Ac site verified with functional assay	References	K-Ac site candidate for deacetylation by Sirt3	References
Ketogenesis	HMGS2	HMGCoA synthase 2	K310, K447, K473 ↓	Shimazu et al., 2010	K46, K333	Rardin et al., 2013a
	HMGL	HMGCoA lyase			K324	Rardin et al., 2013a
	ACAT1	AcetylCoA acetyl transferase 1	K260, K265 ↓	Still et al., 2013	K260	Hebert et al., 2013; Rardin et al., 2013a
Acetate metabolism	AceCS1	AcetylCoA synthetase 1	K661 ↓	Hallows et al., 2006		
	AceCS2	AcetylCoA synthetase 2	K642(h), K635 ↓	Hallows et al., 2006; Schwer et al., 2006		
Urea cycle	OTC	Ornithine transcarbamylase	K88 ↓	Hallows et al., 2011; Yu et al., 2009	K88, K289, K275	Hallows et al., 2011; Hebert et al., 2013; Rardin et al., 2013a
	CPS1	Carbamoylphosphate synthase 1			K1356, K1486	Hebert et al., 2013; Rardin et al., 2013a
	ASL	Argininosuccinate lyase	K288 (h) ↓	Zhao et al., 2010		

Arrows indicate whether the enzyme is activated (up) or inactivated by acetylation (down). Numbering of acetylation sites refers to mouse unless otherwise noted (h, human; r, rat).

is acetylation of ornithine transcarbamylase (Otc) at K88 (see also **Table 1** for more examples). The mechanisms underlying enzyme inhibition seem to vary by protein. In certain cases acetylation at sites within or adjacent to the active site prevents catalytic steps such as substrate binding (Lundby et al., 2012; Bharathi et al., 2013; Hebert et al., 2013; Still et al., 2013). In others, such as Pepck1, acetylation is reported to promote degradation of the enzyme (Zhao et al., 2010; Jiang et al., 2011).

STOICHIOMETRY AND DYNAMICS OF PROTEIN LYSINE ACETYLATION IN MAMMALIAN MODELS

Proteomic studies have certainly revealed many mitochondrial acetyl-proteins, but is the extent or stoichiometry of acetylation sufficient to meaningfully affect their function? Fortunately, MS can provide clues on this front as well. For example, using enzyme immunoprecipitation, coupled with a quantitative proteomic strategy that used isobaric tags for relative and absolute quantification (iTRAQ)and the appropriate peptide standards, Zhao et al. estimated that the acetylation stoichiometry of malate dehydrogenase (Mdh2) and Enoyl-CoA Hydratase/Hydroxyacyl-CoA dehydrogenase (Ehhadh), in HEK293T cells ranges from 7% up to 47% depending on the specific site (Zhao et al., 2010). Moreover, acetylation levels increased substantially in response to treatment with trichostatin A and nicotinamide with profound effects on enzyme activity. It is important to note, that interventions such as knocking out genes or inducing acute and chronic changes in nutrition, can lead to changes in protein expression that can confound acetylation site quantification. In these cases, it is important to show that the abundance of the protein of interest does not vary significantly between groups (for example the abundance of Otc, Mdh2, Lcad, Sdha, and Hmgcs is similar between wild-type and Sirt3$^{-/-}$ samples; Rardin et al., 2013a). Another common approach is to normalize the number of spectra obtained for an acetylated site after immunoenrichment to the total number of spectra obtained for that site in non-enriched samples. This approach was used to quantify changes in acetyllysine site occupancy under conditions of CR, fasting and refeeding or chronic overnutrition (Hebert et al., 2013; Still et al., 2013). Using normalization, it has also been estimated that the acetyllysine occupancy at K147 in aldolase reaches approximately 30% in the liver (Lundby et al., 2012).

A key corollary is: how long does it take to achieve appreciable changes in acetylation levels? Are the rates of acetylation and deacetylation comparable to those of phosphorylation and dephosphorylation? It has been suggested that factors influencing these dynamics may include intrinsic characteristics of the lysine residue being modified (i.e., position in the tertiary structure, making it more or less accessible for acetylation/deacetylation), or extrinsic factors such as the quantity and activity of deacetylases, or the availability of acetyl-CoA and matrix pH (Wagner and Payne, 2013; Pougovkina et al., 2014a). A survey of the literature reveals that in cell culture, widespread changes in acetylation are reported after 24 h of exposure to deacetylase inhibitors (Choudhary et al., 2009). Similarly, robust acetylation of Otc (see also **Table 1**) in cell culture is reported after 16 h of exposure to deacetylase inhibitor nicotinamide (Hallows et al., 2011). In liver

mitochondria, changes in acetylation are detected as early as 2 h of acute re-feeding (Still et al., 2013). Fasting for 12 or 24 h also alters the acetylation pattern in MLM (Kim et al., 2006; Hirschey et al., 2010). Likewise acetylation is modulated by modest but long-term chronic changes in feeding. Chronic protocols such as 2 months of CR, 2.5 months of overnutrition or 3 months of high-fat feeding all yield changes in the acetylation patterns in liver mitochondrial proteins (Hirschey et al., 2011; Hebert et al., 2013; Still et al., 2013).

The deacetylase activity and protein levels of Sirt3 are regulated by nutrient availability. During a 16-h fast, Sirt3-dependent deacetylation occurs (Still et al., 2013), yet no changes in protein abundance are detected, suggesting that short-term fasting elicits Sirt3's enzymatic activity, likely through transient increase of NAD^+ availability. However, after 24 h of fasting, Sirt3 protein levels significantly increase, through mechanisms requiring the transcriptional activator Pgc-1α (Hirschey et al., 2010). Furthermore, elevated protein levels of Sirt3 in the liver are also found after 2 or 3 months of CR (Hallows et al., 2011; Hebert et al., 2013). On the other hand, Sirt3 protein levels decline in liver mitochondria during the second or third month of high fat feeding (Hirschey et al., 2011). Thus, Sirt3 can respond to both acute and chronic changes in nutrition and its activation or inhibition can greatly influence the acetylation dynamics in mitochondria.

MOTIFS IN SUBSTRATE SITES THAT MIGHT SIGNAL ACETYLATION AND DE-ACETYLATION

Current efforts are also focused in the identification of consensus sites that are associated with higher frequency of acetylation and also sites that are subject to deacetylation by Sirt3 in mammalian cells and tissues. Early studies suggested that the presence of tyrosine (Y) or histidine (H) at position +1 is more preferentially identified on acetylated peptides (Kim et al., 2006; Choudhary et al., 2009). A more recent study however, did not identify significant trends for Y or H in position +1 of acetylated peptides in mitochondria (Lundby et al., 2012). In fact, among the different subcellular compartments examined, only nuclear proteins exhibited a strong acetylation motif, consistent with the presence of well-known acetyl-transferases in the nucleus. Therefore, with regard to mitochondria, a universal acetylation motif is not readily evident. On the other hand, efforts to identify sites that are preferentially de-acetylated by Sirt3 have yielded more consistent results. Thus, it appears that acetyl-K residues located on α-helical regions and flanked by other positively charged residues (i.e., K or R on positions +1 and +2) are more likely to be deacetylated by Sirt3 (Hebert et al., 2013; Rardin et al., 2013a).

PROTEIN ACETYLATION IN THE HEART AND CARDIAC MITOCHONDRIA

We now shift our attention to examine the roles of lysine acetylation in cardiac mitochondria and in systems that may be extrapolated to the cardiac domain. As in other organisms and organs, models ranging from SIRT ablation to CR, from cell biology to proteomics are providing insights that may one day be harnessed for therapeutic benefit.

THE ROLE OF Sirt3 IN THE HEART AND CARDIAC MYOCYTES. CANDIDATE TARGETS FOR DEACETYLATION

Sirt3-deficient mice have been developed by several groups who, collectively have observed elevated acetylation on mitochondrial proteins in tissues lacking the gene (Lombard et al., 2007; Ahn et al., 2008; Yang et al., 2010; Fernandez-Marcos et al., 2012). $Sirt3^{-/-}$ mice have baseline cardiac hypertrophy evident at 4 or 13 months of age (Sundaresan et al., 2009; Hafner et al., 2010), although data from others show no increase in the cardiac mass of $Sirt3^{-/-}$ mice at 5 months of age (Someya et al., 2010). Sirt3 is induced in the heart by exercise or pathologic stimuli and is shown to protect against maladaptive cardiac remodeling and improve survival following pressure overload (Sundaresan et al., 2009; Hafner et al., 2010). It would be reasonable to presume that protection stems from deacetylation of mitochondrial substrates, yet initial evidence in cardiac cells has highlighted putative roles of Sirt3 outside mitochondria. Specifically, in neonatal rat ventricular myocytes (NRVMs), Sirt3 deacetylates nuclear protein Ku70, prevents mitochondrial translocation of the proapoptotic protein Bax, and enhances tolerance against H_2O_2 (Sundaresan et al., 2008). Moreover, Sirt3 deacetylates transcription factor Foxo3a, which increases the expression of manganese-dependent superoxide dismutase (MnSOD, Sod2) that, in turn, diminishes the accumulation of superoxide in mitochondria (Sundaresan et al., 2009). Data notwithstanding, it is still debatable whether Sirt3 is a *bona fide* regulator of Ku70 and Foxo3a, or whether enforced expression of Sirt3 accounts for the observed extra-mitochondrial activity (Bao et al., 2010). Furthermore, a quick survey in the acetylome database (http://cpr1.sund.ku.dk/cgi-bin/PTM.pl) failed to retrieve any acetylated peptides for Ku70 or Foxo3a in the heart, suggesting that the frequency of this modification might be below the detection limits of the MS/MS approach.

Acetylation of Sod2 is of considerable interest, since the enzyme is a critical component of mitochondrial antioxidant defenses and Sod2 deficiency exacerbates cardiac injury *in vivo* (Lebovitz et al., 1996). Although Sod2 acetylation in the heart has yet to be characterized fully, there is consensus among studies in liver, mouse embryo fibroblasts (MEFs), and HEK cells demonstrating that Sod2 activity is regulated via reversible acetylation and is controlled by Sirt3 (Qiu et al., 2010; Tao et al., 2010; Chen et al., 2011). For example, fasting for 36 h decreases the levels of acetylated Sod2 in the liver without reducing total protein levels and that regulation requires the presence of Sirt3 (Tao et al., 2010). Similarly, CR for 6 months (30% reduction in caloric intake) reduces the acetylation of Sod2 in the liver in a Sirt3-dependent fashion (Qiu et al., 2010). Moreover, Sirt3 is demonstrated to interact with and deacetylate Sod2 in MEFs and HEK293 cells, and in the absence of Sirt3, Sod2 exhibits increased acetylation and inhibited enzymatic activity *in vitro* (Qiu et al., 2010; Tao et al., 2010; Chen et al., 2011). Interestingly, each of these studies identified different lysine residues as the pivotal sites for regulation of Sod2 via reversible acetylation, including K53, K68 K89 and K122 (see also **Table 2**). Activation of Sod2 in the liver increases the ratio of [GSH:GSSG] (an index of antioxidant capacity) while decreasing indices of oxidative stress including hydroxynonenal, protein carbonylation and mitochondrial superoxide production (Qiu et al., 2010; Tao et al., 2010). Furthermore,

Table 2 | Mitochondrial protein acetylation, regulatory sites and significance in cardiac physiology and pathophysiology.

Mitochondrial process	Protein/ enzyme	Regulatory K-Ac site(s)	Conditions affecting acetylation	Experimental system	References	K-Ac sites in heart mitochondria acetylomes		
						Rat (Lundby et al., 2012)	Mouse (Nguyen et al., 2013)	Guinea pig (Foster et al., 2013)
Substrate metabolism	Acaa2					K25, K137, K270	K25, K137, K270	K25, K270
	Lcad		HF ↑	Heart	Grillon et al., 2012	K81, K254, K279	K81, K254, K279	K81, K254, K279
	Hadha		CypD deficiency ↑	Heart	Nguyen et al., 2013	K436,K505,K728	K505, K728	K505, K728
	Hadhb		IPC ↑	Heart	Nadtochiy et al., 2011	K73, K349	K73, K202	K73, K202, K349
			CypD deficiency ↑	Heart	Nguyen et al., 2013			
TCA cycle /ETC /ATP synthesis	Mdh2		HF ↑	Heart	Grillon et al., 2012	K105,K165,K328	K105,K165, K328	K165, K328
	Sdha		CR ↑	Heart	Shinmura et al., 2011	K498,K550,K633	K498,K550, K633	K498, K550
			Ndufs4 deficiency ↑	Heart	Karamanlidis et al., 2013			
	Aco2		CypD deficiency ↑	Heart	Nguyen et al., 2013	K50, K144, K517	K50, K144, K517	K50, K517
	Ndufa9		Sirt3 deficiency ↑	Liver, MEFs	Ahn et al., 2008	K189	K189	K189
			Ndufs4 deficiency ↑	Heart	Karamanlidis et al., 2013			
			Frataxin deficiency ↑	Heart	Wagner et al., 2012			
	Ndufs1		CR ↓	Heart	Shinmura et al., 2011	K450, K499	K450, K467	K450, K467
	Ndufv1		IPC ↓	Heart	Nadtochiy et al., 2011	K81,K104, K401	K81,K104, K401	K104
			HF ↑	Heart	Grillon et al., 2012			
	Uqcrc1		HF ↑	Heart	Grillon et al., 2012	K85, K111, K138	K85, K111, K138	K85, K111, K138
	Uqcrfs1		CR ↓	Heart	Shinmura et al., 2011	K46, K168, K172	K163, K168	K46
	Atp5a1		CR ↑	Heart	Shinmura et al., 2011	K427,K531,K539	K427,K531, K539	K427,K531, K539
	Atp5b		HF ↑	Heart	Grillon et al., 2012	K133,K159,K259	K133, K259	K133, K259
	Ckmt2		IPC ↓	Heart	Nadtochiy et al., 2011	K276,K292,K355	K276	K276
			HF ↑	Heart	Grillon et al., 2012			
Redox balance	Sod2	K122	Fasting ↓, Sirt3 defic. ↑	Liver, MEFs	Tao et al., 2010	K68, K114, K122	K68, K114	K68, K122, K130
		K53, K89	CR ↓, Sirt3 deficiency ↑	Liver, MEFs	Qiu et al., 2010			
		K68	Nutrient starvation ↓	HEK293	Chen et al., 2011			
	Idh2		CR ↓	Liver, Brain,	Someya et al., 2010	K155,K180,K199	K155,K180, K413	K155, K180,K199
			IPC ↓	Heart	Nadtochiy et al., 2011			
			CypD deficiency ↑	Heart	Nguyen et al., 2013			
	Prdx5					K82, K145, K186	K79, K81, K82	K86
	Txnrd2					K137, K153	K153	K153
Permeability transition	CypD	K166	Switch from Glc. to Gal. ↓	HeLa	Shulga et al., 2010	K66, K166, K174	K66, K174	K174
		K166	Sirt3 expression ↓	Heart, HEK293	Hafner et al., 2010			

(Continued)

Table 2 | Continued

Mitochondrial process	Protein/ enzyme	Regulatory K-Ac site(s)	Conditions affecting acetylation	Experimental system	References	K-Ac sites in heart mitochondria acetylomes		
						Rat (Lundby et al., 2012)	**Mouse** (Nguyen et al., 2013)	**Guinea pig** (Foster et al., 2013)
Membrane fusion	Opa1	K926, K931	Sirt3 deficiency ↑ cardiac stress ↑	Heart, MEFs	Samant et al., 2013	Not found	Not found	Not found
Translation	Mrpl10	K124, K162, K196	Sirt3 expression ↓	Liver, HEK293	Yang et al., 2010	Not found	Not found	Not found

IPC, ischemic preconditioning; CR, calorie restriction; HF, heart failure; Arrows indicate whether acetylation is increased (up) or decreased (down) by the different experimental conditions. K-Ac sites are reported according to mouse numbering; due to space limitations not all acetylated sites are shown, instead whenever possible, the conserved sites across species are shown; a blank cell indicates that no data are currently available.

Sod2 deacetylation curtails apoptosis in the liver and ameliorates cell death induced by paraquat (an inducer of oxidative stress). In MEFs, the expression of Sod2 deacetylation-mimetic mutants (i.e., K-to-R) abrogates mitochondrial superoxide production (Qiu et al., 2010; Tao et al., 2010). Thus, the activity of Sod2 depends on its acetylation state and it remains to be determined whether a similar mechanism is operable in the heart. Along these lines, it has been very recently reported that partial deficiency in Sirt3 is associated with lower Sod2 enzymatic activity in mouse hearts (Porter et al., 2014). These Sirt3 haploinsufficient hearts also demonstrated increased susceptibility to ischemia/reperfusion (I/R) injury (Porter et al., 2014).

REGULATION OF PROTEIN ACETYLATION DURING CALORIE RESTRICTION IN NEURONS AND IN HEART

Like cardiac myocytes, neurons are post-mitotic cells with increased energy demands and heightened risk for oxidative damage. CR (25% reduction in calorie intake for 10 months) protected against neuronal death and hearing loss by activating antioxidant mechanisms in mice (Someya et al., 2010). A similar approach (40% reduction in calorie intake for 6 months) was also used to test the effects of CR in cardiac I/R injury in rats (Shinmura et al., 2011). In neurons, the protective effects of CR are mediated by increasing protein levels and activity of Sirt3 (Someya et al., 2010). In the heart, CR increases the overall mitochondrial sirtuin activity, although the protein levels of Sirt3 do not change significantly (Shinmura et al., 2011). In neurons, the anti-aging effects of CR implicate the deacetylation and activation of mitochondrial NADP-dependent isocitrate dehydrogenase 2 (Idh2; activation of this enzyme increases [NADPH:NADP+] ratio, which drives thiol-dependent antioxidant defenses). Likewise, CR up-regulates NADPH concentration and [GSH:GSSG] ratios in neuronal and liver mitochondria. Moreover, Idh2 maintains high levels of [NADPH] in HEK cells and protects against cell death induced by oxidants such as peroxide and menadione (Someya et al., 2010). In the case of heart, CR has protective effects in some but not all post-I/R indices of mitochondrial function. For example, oxygen consumption rates and mitochondrial H_2O_2 levels are largely unchanged by CR, although mitochondrial swelling due to Ca^{2+}–overload is ameliorated (Shinmura et al., 2011). Acetylation of Idh2 was not studied in the CR-treated heart, but acetylation in a number of other proteins changed (see CR, **Table 2**). Notably, during this regimen, some mitochondrial proteins were deacetylated (Ndufs1, Uqcrfs1, and Sdha) whereas others became hyperacetylated (Atp5a1) (Shinmura et al., 2011). These targets offer starting points for further research into the cardioprotective mechanisms of CR.

REGULATION OF CARDIAC PROTEIN ACETYLATION IN HEART FAILURE AND ISCHEMIC PRECONDITIONING

Beyond cardiac I/R injury, mitochondrial protein acetylation has also been studied in the context of ischemic preconditioning (IPC), and experimental heart failure (HF). In the case of IPC, acetylation of mitochondrial proteins can be either upregulated or downregulated, a situation somewhat similar to that observed with CR-treated hearts. For example, the acetylation

of Hadhb increases whereas that of Ndufv1 decreases during IPC (Nadtochiy et al., 2011) (see also IPC, **Table 2**). In HF however, there appears to be a more pronounced trend toward increased acetylation. In a study where rats developed HF at 7.5 or 18 months of age, it was determined that 41 or 66 cardiac proteins respectively exhibited increased acetylation (Grillon et al., 2012). Examples of acetylated proteins include Ndufv1, Uqcrc1, Mdh2, and Ckmt2 (see also **Table 2**) (Grillon et al., 2012). Increased mitochondrial protein acetylation is also shown in a murine model of diabetic cardiomyopathy (Vadvalkar et al., 2013). Provided that appropriate normalization is performed for changes in protein abundance, it can be suggested that HF progression is accompanied by increased mitochondrial protein acetylation. Notably, Sirt3 protein levels decrease in failing rat hearts or when H9C2 cardiomyocytes are subjected to oxidative stress (Grillon et al., 2012; Chen et al., 2013). Finally, notwithstanding the protein remodeling that might compromise mitochondrial function during HF progression, it has been shown that acute induction of acetylation chemically with acetic anhydride is sufficient to adversely impact mitochondrial function, specifically lowering state III respiration and elevating ROS production (Vadvalkar et al., 2013). Mitochondrial protein deacetylation might also be necessary for successful cardiac IPC, however the bulk of the current data center on important regulators of IPC in the cytosol. This is supported by the finding that a sirtuin inhibitor, splitomycin, negates the post-ischemic recovery of preconditioned hearts (Nadtochiy et al., 2011). Since splitomycin targets Sirt1 and is likely to block deacetylation of cytoplasmic proteins, it remains an open question whether deacetylation of mitochondrial proteins and the relevant sirtuin Sirt3 is required for effective IPC. It would therefore seem that accumulating evidence supports the argument that mitochondrial lysine acetylation in the heart is harmful. Alternatively, it has also been suggested that acetylcarnitine treatment may alleviate age-related mitochondrial dysfunction, in part, via acetylation and activation of mitochondrially-encoded gene expression (Rosca et al., 2009).

LYSINE ACETYLATION ON CYCLOPHILIN D

Cyclophilin D (CypD) operates as a mitochondrial chaperone with *cis/trans* (C/T) isomerase activity but is best known as a modulator of acute necrotic cell death through the mitochondrial permeability transition (MPT). Studies in cancer cell lines and in HEK cells have suggested that acetylated CypD can more readily sensitize MPT (Hafner et al., 2010; Shulga et al., 2010). In HeLa cells growing in glucose, CypD is acetylated and has enhanced C/T isomerase activity whereas in cells growing in galactose, CypD is deacetylated by Sirt3 and its C/T isomerase activity declines (Shulga et al., 2010). The interaction between Sirt3 and CypD and the deacetylation of the latter is also documented in HEK cells (Hafner et al., 2010). K166 in CypD is identified as a critical site for deacetylation by Sirt3 (**Table 2**). This site is important for C/T isomerase activity and K166's acetylation may increase CypD's capacity to interact with other protein partners to induce MPT and reduce mitochondrial respiration rates (Shulga et al., 2010). Together, these studies provide an example where increased acetylation becomes detrimental, not by inhibiting enzymatic activity or protein complexes but by activating a protein to adversely affect mitochondrial functions. Nevertheless, it is increasingly recognized that not all CypD functions are detrimental to mitochondria. For example, CypD may be involved in the release of excess Ca^{2+} from mitochondria (Elrod et al., 2010).

OTHER CARDIAC MODELS ASSOCIATED WITH INCREASED PROTEIN ACETYLATION

Apart from ablation of Sirt3 (Lombard et al., 2007; Ahn et al., 2008; Fernandez-Marcos et al., 2012), a protein with documented deacetylase activity, other proteins have also been shown to have an impact on mitochondrial lysine acetylation levels, among them, Ndufs4, frataxin (frtxn), and CypD (Wagner et al., 2012; Karamanlidis et al., 2013; Nguyen et al., 2013). Ndufs4 is a subunit of complex I (NADH dehydrogenase) and its deletion in cardiac myocytes renders mitochondria deficient in complex I-driven respiration (Karamanlidis et al., 2013). Owing to complex I deficiency, the generation of NAD^+ from NADH is blocked and the [NADH:NAD^+] ratio is elevated. In frataxin deficiency, iron-sulfur cluster biogenesis and respiratory complex formation are impaired, again leading to elevated [NADH:NAD^+] ratio (Wagner et al., 2012). In the case of CypD deficiency, mitochondria exhibit increased activity of matrix dehydrogenase complexes pyruvate dehydrogenase complex (PDHc) and KGDHc (Elrod et al., 2010) which can also elevate the [NADH:NAD^+] ratio. Therefore, these gene deficiencies are characterized by increased [NADH:NAD^+] ratios which would be expected to inhibit Sirt3, whose deacetylase activity is dependent on NAD+ (**Figure 1**) (Tanner et al., 2000). Consistently, mitochondria isolated from Ndufs4$^{-/-}$, frtxn$^{-/-}$, or CypD$^{-/-}$ hearts have altered [NADH/NAD^+] ratios and hyperacetylated proteins, despite unchanged Sirt3 protein levels (Wagner et al., 2012; Karamanlidis et al., 2013; Nguyen et al., 2013). It bears noting, however, that high NADH in these models could also lead to feedback inhibition of the TCA cycle and accumulation of acetyl-CoA. In turn, this might well contribute to hyperacetylation independently of Sirt activity.

Examples of proteins whose acetylation is increased in Ndufs4$^{-/-}$, frtxn$^{-/-}$, and CypD$^{-/-}$ mitochondria are shown in **Table 2**. Sirt3 localizes to complex I where it deacetylates subunit Ndufa9, but fails to do so after treatment with rotenone (a complex I inhibitor) (Ahn et al., 2008). Furthermore, rotenone treatment increases mitochondrial protein acetylation to an extent similar to that observed when mitochondria are treated with nicotinamide (a sirtuin inhibitor) (Karamanlidis et al., 2013). These findings illustrate that even short term inhibition of complex I can increase protein acetylation in cardiac mitochondria. Likewise, with respect to CypD action, it would be interesting to test whether cyclosporine A (a CypD inhibitor) yields a similar result in protein acetylation. As for the downstream pathways that are affected in the three genetic models, Ndufs4$^{-/-}$ mitochondria have unchanged [NADPH:$NADP^+$] ratios, unchanged aconitase activity and unchanged acetylation levels of Sod2 all consistent with normal redox defense mechanisms (Karamanlidis et al., 2013). On the other hand CypD$^{-/-}$ mitochondria have increased acetylation of Idh2 consistent with reduced capacity to counteract redox stress (Nguyen et al., 2013). Frtxn$^{-/-}$ hearts

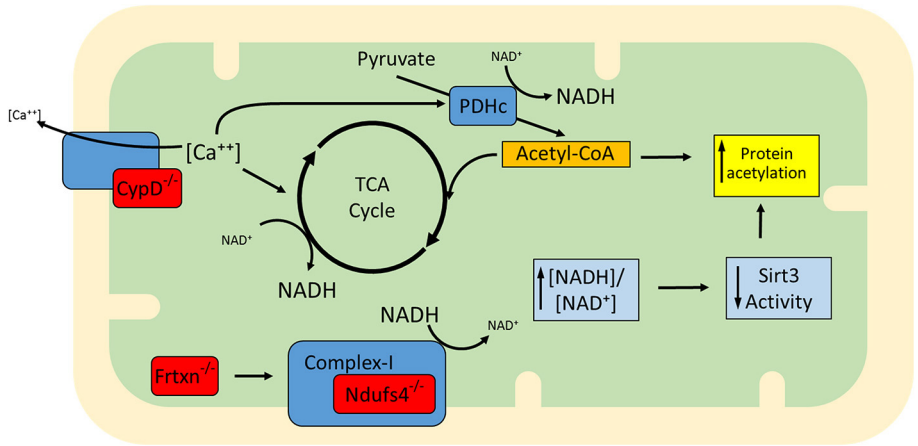

FIGURE 1 | Proposed model for the increased mitochondrial protein acetylation detected in the absence of Ndufs4, CypD, and frataxin (gene deficiencies shown in red). CypD facilitates Ca^{++} efflux and when absent, mitochondria exhibit higher than normal [Ca^{++}]. This upregulates matrix dehydrogenases (e.g., TCA cycle dehydrogenases and PDH). Dehydrogenase activation enhances generation of NADH, altering the balance to higher ratios of [NADH:NAD^+]. Complex I normally converts NADH back to NAD^+ but in the absence of Ndufs4 this conversion is blocked. Defects in complex I activity may also arise in the absence of Fe-S cluster biogenesis, a mitochondrial process regulated by frataxin. Although described independently (Wagner et al., 2012; Karamanlidis et al., 2013; Nguyen et al., 2013), the three conditions are similar in that they exhibit an increase in protein acetylation which may be at least in part attributable to elevation of [NADH:NAD^+] ratios and lowering of Sirt3 activity. It remains to be determined whether mechanisms other than Sirt3 inhibition (e.g., elevation of acetyl-CoA) also contribute to increased acetylation in these models.

have high levels of protein carbonylation, also suggesting reduced capacity to counteract redox stress (Wagner et al., 2012). Finally, at the level of cardiac function, Ndufs4$^{-/-}$ hearts have normal function at baseline which declines following pressure overload (Karamanlidis et al., 2013). Decreased heart function following pressure overload is also reported for the case of CypD$^{-/-}$ (Elrod et al., 2010), whereas frataxin deficiency is characterized by early stage cardiomyopathy (Puccio et al., 2001). Taken together, these models suggest that increased mitochondrial protein acetylation, caused by either altered [NADH:NAD^+] and/or Acetyl-CoA levels, may be a common mechanism underlying the onset of cardiac dysfunction in response to pathological stressors (**Figure 1**).

CARDIAC ACETYL-LYSINE PROTEOME DATASETS

Though most global acetylome data sets have been obtained from liver, efforts to characterize the broader acetylation profile in the heart have begun. Following fractionation and mitochondrial purification, a gel-free proteomics approach identified about 130 acetylated proteins in the case of guinea pig heart (Foster et al., 2013). Similarly, about 200 acetylated proteins were identified in purified mouse heart mitochondria (Nguyen et al., 2013). Furthermore, proteomics in unfractionated rat hearts yielded about 245 acetylated proteins mapping to the mitochondrion (Lundby et al., 2012). **Table 2** gives a snapshot of some of the mitochondrial substrates detected in the three species, the site(s) of acetylation for each substrate and highlights the functional categories where reversible acetylation might be important (i.e., substrate metabolism, TCA cycle, ETC, ATP synthesis, redox balance, and MPT). It becomes evident that as putative acetylation sites are uncovered by proteomics, there is an increasing demand to verify with functional assays the sites whose reversible acetylation has profound effects in protein function. No doubt, efforts will now turn to investigate how global-scale acetylation profiles are impacted in models of heart disease. Some evidence is already available from models discussed above (e.g., CR, IPC, HF), and large-scale quantitative proteomics will likely uncover new and important details on mitochondrial protein acetylation.

EXPANDING THE SPECTRUM OF PROTEIN LYSINE MODIFICATIONS. SUCCINYLATION AND THE ROLE OF Sirt5

We have previously mentioned how lysine acetylation in mitochondria may be favored by the elevated pH of the matrix and the proximity of proteins to locally-generated acetyl-CoA. Yet acetyl-CoA is only one of several metabolite-CoA species generated in mitochondria (**Figure 2**), which given the same conditions, would also be expected to react with lysine. Again, the combination of technological advancements that led to characterization of the mitochondrial acetylome have been successfully applied for characterizing new modifications such as succinylation and malonylation.

LYSINE SUCCINYLATION IN BACTERIA AND NON-MAMMALIAN EUKARYOTIC CELLS

Protein lysine succinylation (K-Su) results from the reaction of the dicarboxylate compound succinyl-CoA with lysine residues. The addition of a succinyl moiety to lysine adds a mass of 100 Da and imparts a negative charge at physiological pH. Using MS/MS, initial studies in *E.coli* identified 14 succinylated proteins including IcdA (homolog to mammalian Idh2), GapA (homolog to Gapdh) and several ribosome subunits and protein chaperones (homologs to mammalian Hsp60 and Hsp70) (Zhang et al., 2011). More recently, the tally has reached as high as 670 and 990 succinylated proteins in two recent studies (Colak et al., 2013; Weinert et al., 2013c). It is not yet clear what effects K-Su has

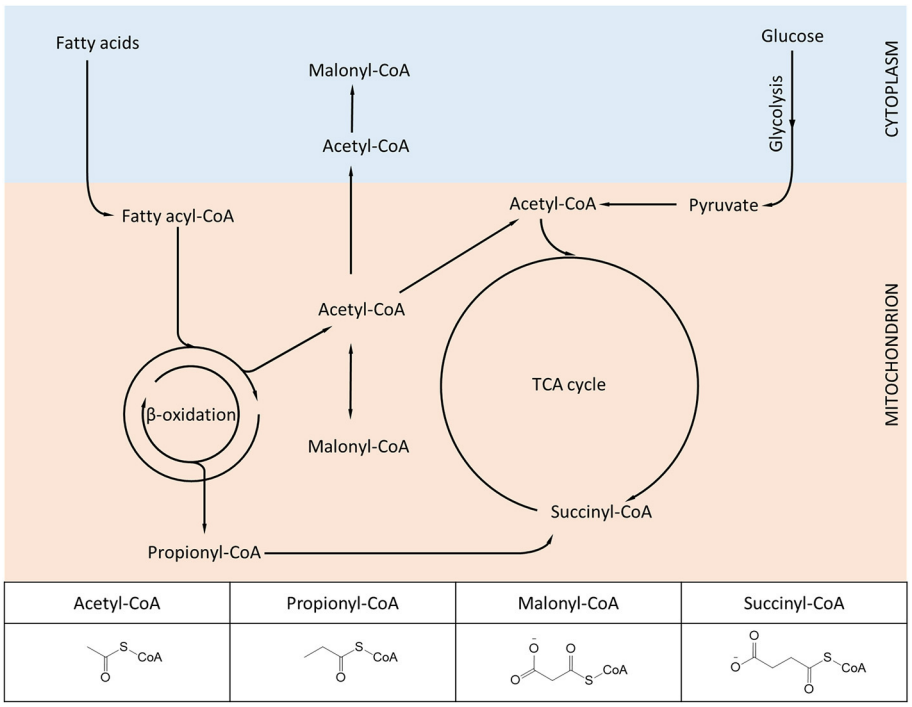

FIGURE 2 | Diagram showing metabolic intermediates produced in mitochondria and utilized in protein lysine modifications. The inset table highlights the structural similarities and differences among the various acyl-CoA species. Lysine acetylation, propionylation, malonylation, and succinylation have been found to dynamically change in mammalian mitochondria, while other acylation variants (e.g., butyrylation, crotonylation) await further experimental characterization.

on the function of protein substrates. To begin addressing these question, site-directed mutagenesis of K to E has been employed to mimic the creation of a K-Su site. In one experiment, a mutant IcdA K242E exhibited decreased rates of NADPH production *in vitro* suggesting that succinylation might be inhibitory for the dehydrogenase activity of IcdA (Zhang et al., 2011).

As in the case of acetylation, growth conditions influence the magnitude of succinylation in *E.coli*. Exposure to sodium succinate for 4 h increases the number of succinylated proteins (Zhang et al., 2011). Similarly, supplementation of minimal growth medium with either pyruvate, succinate or glucose, potently elevates global succinylation levels after 16 h (Colak et al., 2013). Estimates of succinylation stoichiometry on target proteins in *E.coli* are reported but they are only limited to a small number of proteins. The stoichiometry of protein succinylation in *E.coli* appears to range from 0.14 to 34%. For comparison, the stoichiometry of protein acetylation ranges from 0.22 to 73% in the same conditions (Colak et al., 2013). Succinylation (K-Su) appears to overlap considerably with acetylation (K-Ac). In *E.coli* grown in 0.8% glucose, K- Su and K-Ac sites overlapped by 39.3% (Colak et al., 2013), while in *E.coli* grown in 0.2% glucose, K-Su and K-Ac sites overlapped by 18.6% (Weinert et al., 2013c). It is not yet clear whether competition between acetyl- and succinyl-moieties for the same K site offers an additional regulatory level for controlling the activity of the protein substrate.

Bacteria contain a sirtuin-like protein (CobB) that functions as a protein deacetylase (Starai et al., 2002; Zhao et al., 2004; Weinert et al., 2013a). It was nevertheless hypothesized that CobB might have a broader specificity and function as a protein desuccinylase. Using wild-type and CobB-deficient bacteria, two studies reached different conclusions. In one study, the deletion of CobB did not induce considerable increases in global levels of protein succinylation (Weinert et al., 2013c). However, another study demonstrated that loss of CobB induced significant increases in global protein succinylation suggesting that CobB opposes protein succinylation (Colak et al., 2013). *In vitro* assays further confirmed CobB's ability to desuccinylate substrate peptides and highlighted residues within the protein that might be important for interacting with the K-Su substrate (Colak et al., 2013). It should be noted that the growth conditions in the two studies were different (i.e., in the presence of 0.2 or 0.8% glucose), which might in part be responsible for the discrepancies obtained regarding the desuccinylase activity of CobB. Once the role of CobB in desuccinylation is independently corroborated, it would be interesting to determine which K-Su substrate sites are subject to regulation by this enzyme and whether the enzyme can be regulated to preferentially distinguish between K-Su and K-Ac sites.

In addition to prokaryotic cells, lysine succinylation was identified in yeast (*S.cerevisiae*) and SL2 cells (*D.melanogaster*) (Zhang et al., 2011; Xie et al., 2012; Weinert et al., 2013c), suggesting that this PTM is evolutionarily conserved. A proteome-wide study in yeast identified 474 succinylated proteins (Weinert et al., 2013c). Notwithstanding that succinyl-CoA, the primary metabolite required for succinylation, is produced inside

mitochondria (**Figure 2**) only a small fraction of proteins identified in the yeast succinylome (61 out of 474) map to mitochondria, while the majority are cytoplasmic and nuclear (Xie et al., 2012; Weinert et al., 2013c). Altering the availability of succinyl-CoA (by genetically ablating specific enzymes of the TCA cycle) affects the global succinylation pattern in yeast, suggesting that succinyl-CoA used for succinylation comes from mitochondria (Weinert et al., 2013c). Nevertheless, it is not yet clear how succinyl-CoA is exported to the cytoplasm. As in prokaryotic cells, K-Su and K-Ac sites tend to overlap, although in the case of yeast this is less pronounced (∼16%) (Weinert et al., 2013c). While absolute stoichiometries of protein succinylation remain to be determined, the current evidence suggests that for most yeast proteins this is low, or at least much lower than the stoichiometries of phosphorylation. This evidence suggests that lysine succinylation in yeast operates as a low-grade PTM, although it might prove important for protein regulation under conditions that have yet to be identified. Another open question is whether yeast protein succinylation is subject to regulation by *Sir2*.

REGULATION OF PROTEIN LYSINE SUCCINYLATION BY Sirt5

Almost simultaneously, two groups made the seminal discovery that Sirt5, a mitochondrial sirtuin with only marginal deacetylase activity, had the ability to function as a *desuccinylase* (Du et al., 2011; Peng et al., 2011). Using a thin layer chromatography assay to monitor conversion of radiolabeled NAD^+ to acyl-ADP-ribose, a hallmark of sirtuin activity, it was shown that high purity SIRT5 displayed activity toward chemically synthesized succinyl-lysine peptides (Du et al., 2011). Desuccinylation by Sirt5 was confirmed by HPLC-MS or fluorescent techniques and Sirt5's activity was sensitive to inhibition by nicotinamide (Du et al., 2011; Peng et al., 2011). A crystal structure of Sirt5, in complex with a succinylated peptide, indicated that residues Y102 and R105 are important for interactions with the negatively-charged carboxyl group of the succinylated lysine (Du et al., 2011). Furthermore, R105 is suggested to confer increased sensitivity to inhibition by nicotinamide (Fischer et al., 2012). Another Sirt5 residue, H158, is important for general sirtuin catalytic activity (Schwer et al., 2002; Nakagawa et al., 2009) and, consistently, a mutant H158Y exhibits no desuccinylase activity (Du et al., 2011; Peng et al., 2011). By immobilizing Sirt5 on a column and subjecting a bovine liver mitochondrial extract to affinity purification, the first substrates of Sirt5 were identified, including Got2 (glutamate oxaloacetate transaminase 2) and Hmgcs2 (HMG-CoA synthetase 2) (Du et al., 2011). The finding that Sirt5 operates as a protein lysine desuccinylase was instrumental in characterizing the functions and regulation of this PTM, as discussed in the section below. Of note, Sirt5 exhibits broader specificity for modified lysines and in addition to desuccinylation, it can also perform demalonylation and deglutarylation (Du et al., 2011; Peng et al., 2011; Tan et al., 2014) (**Figure 3**).

CHARACTERIZING PROTEIN LYSINE SUCCINYLATION IN MAMMALIAN EXPERIMENTAL SYSTEMS

The study of succinylation in mammalian cells encompasses HeLa, HepG2, HEK293, and wild-type or Sirt5-deficient MEFs

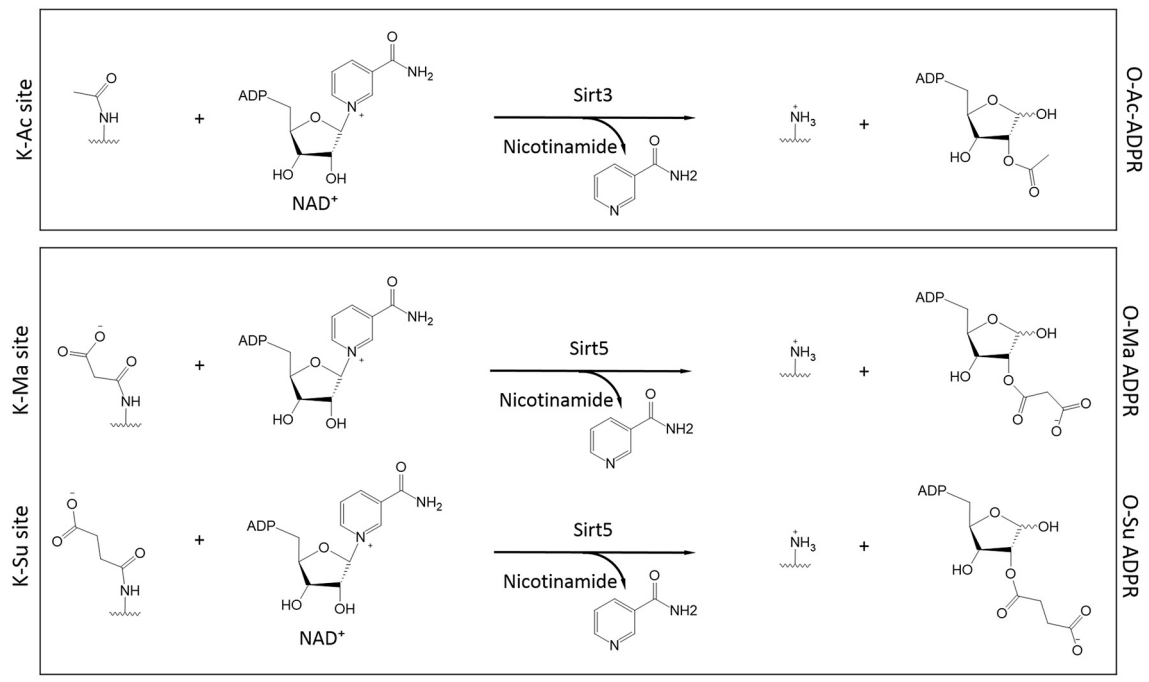

FIGURE 3 | Role of Sirt3 and Sirt5 in the catalysis of protein deacylation in the presence of NAD^+. Sirt3 has specificity for acetylated lysines (K-Ac) whereas Sirt5 has broader specificity and it can accommodate malonylated and succinylated substrates (K-Ma and K-Su respectively). In all cases, NAD^+ is consumed to release nicotinamide while the ADP-Ribose (ADPR) moiety serves as the final acceptor of the acyl group (O-Ac-, O-Ma-, and O-Su-ADPR respectively). The acyl-modified and de-modified site is also shown.

(Peng et al., 2011; Park et al., 2013; Rardin et al., 2013b; Weinert et al., 2013c). Among tissues, succinylation has been studied primarily in the liver (Du et al., 2011; Peng et al., 2011; Park et al., 2013; Rardin et al., 2013b; Weinert et al., 2013c; Yu et al., 2013; Buler et al., 2014) and skeletal muscle (Rardin et al., 2013b; Yu et al., 2013). Sirt5$^{-/-}$ mice have been used in several studies (Lombard et al., 2007; Du et al., 2011; Peng et al., 2011; Park et al., 2013; Rardin et al., 2013b; Yu et al., 2013) and in many cases mitochondria have been the focal point (Fritz et al., 2013; Park et al., 2013; Rardin et al., 2013b; Buler et al., 2014). To assess the mammalian succinylome, three independent studies have employed polyclonal succinyl-specific antibodies to enrich for succinylated peptides that were subsequently analyzed by MS/MS approaches (Park et al., 2013; Rardin et al., 2013b; Weinert et al., 2013c). Succinylated proteins were identified with a false-discovery rate (fdr) less than 1%. The focus of each of the studies was (i) mouse liver and HeLa cells (Weinert et al., 2013c), (ii) MLM from wild-type or Sirt5 KO mice, (Rardin et al., 2013b), and (iii) wild-type or Sirt5 KO MEFs (Park et al., 2013). In MEFs, a total of 505 succinylated proteins were identified (Park et al., 2013), whereas in HeLa cells this number reached 738, of which 272 are known mitochondrial proteins. Likewise, in whole mouse liver samples, 750 succinylated proteins were recovered, of which 310 are mitochondrial (Weinert et al., 2013c). Lastly, the analysis of purified MLM yielded 252 succinylated proteins (Rardin et al., 2013b). Collectively, these findings show that lysine succinylation is a frequent modification in mammalian cells whose breadth and cellular localization rival that of acetylation, which prompts the question: do these PTMs target common sites? K-Su/K-Ac overlap appears to be low (∼8–10%) in MEFS as well as HeLa cells (12.6%) (Park et al., 2013; Weinert et al., 2013c). By contrast, K-Su/K-Ac overlap was higher in mouse liver (24%) and reached a maximum (38.5%) in purified liver mitochondria (Rardin et al., 2013b; Weinert et al., 2013c). Given that the highest overlap is observed in mitochondria, the variations observed between cell types might reflect differences in their mitochondrial content. Moreover, the extensive overlap inside mitochondria is, again, consistent with the fact that these proteins are in proximity to the source of the acetyl- and succinyl-CoA metabolites (Wagner and Payne, 2013).

DYNAMICS AND STOICHIOMETRY OF LYSINE SUCCINYLATION

Elucidating the rates with which proteins are succinylated/desuccinylated could lead to better predictions of how this PTM regulates downstream targets. In-depth kinetic studies specifically tailored to assess the dynamics of succinylation in different models and conditions are not yet available. Nevertheless, data from cell-free systems and *in vivo* show that succinylation progresses over the course of hours to days. For example, chemical (i.e., non-enzymatic) succinylation of BSA occurs within 3–6 h (Wagner and Payne, 2013; Weinert et al., 2013c). In cells, pulse-chase experiments with deuterated sodium succinate (D4) determined that succinylation gradually increases within 8–24 h (Xie et al., 2012; Park et al., 2013). In mice, global protein succinylation in liver increases at 48 h of fasting (Park et al., 2013). Moreover, mitochondrial protein succinylation in liver is reduced over the course of several weeks of ethanol exposure (Fritz et al., 2013). While these findings show that succinylation dynamics are slow, they do not rule out the possibility of faster changes under specific conditions. Future studies assessing the kinetics of succinylation/desuccinylation will need to account for the levels and activity of Sirt5 as well as the availability of succinyl-CoA.

Absolute succinylation stoichiometries were assessed in MEFs by a quantitative MS method that uses stable isotope labeling in cells (SILAC) (Park et al., 2013). The majority of quantified proteins had succinylation stoichiometries equal to or less than 10% (Park et al., 2013). Similar stoichiometry trends were obtained for succinylated sites; the site most heavily succinylated (Hspd1 K352) had a stoichiometry close to 80% but the majority of sites had stoichiometries around 15% or below (Park et al., 2013). For reference, the phosphorylation site stoichiometry in dividing HeLa cells is usually above 75% for most substrates (Olsen et al., 2010). Efforts to identify a consensus target sequence, among all succinylation sites, that might imply an enzyme-mediated PTM, have revealed no clear trends (Park et al., 2013; Rardin et al., 2013b; Weinert et al., 2013c). However, examination of sites desuccinylated by Sirt5 reveals a putative target motif in which succinyl lysine is flanked by serines and/or threonines, i.e., (S/T)-K-(S/R) (Rardin et al., 2013b).

EFFECTS OF Sirt5 ON CELL-WIDE AND MITOCHONDRIAL SUCCINYLATION

Succinyl-CoA levels and Sirt5 activity have emerged as the main regulators of protein succinylation and desuccinylation respectively. Analysis of Sirt5 deficient tissues and cells (liver, skeletal muscle, kidney, primary hepatocytes, and MEFs) shows that loss of Sirt5 increases global succinylation, consistent with the proposed role of Sirt5 in repressing succinylation (Peng et al., 2011; Park et al., 2013; Rardin et al., 2013b; Yu et al., 2013). Moreover, Sirt5 deficiency has no effect on global acetylation levels (Peng et al., 2011; Park et al., 2013; Rardin et al., 2013b), nor did Sirt3 deficiency affect global succinylation (Fritz et al., 2013). To identify the *in vivo* substrates of Sirt5, two studies characterized the succinylome in MEFs and MLM with or without Sirt5 (Park et al., 2013; Rardin et al., 2013b). Both studies detect Sirt5 in mitochondria and the cytoplasm, implying that Sirt5 targets both compartments. Furthermore, one study identified about 120 extramitochondrial proteins whose succinylation was regulated by Sirt5 (Park et al., 2013). On the other hand, a separate study did not detect significant succinylation in the cytoplasm of liver extracts or in MEFs by western blot, although it detected prolific succinylation in purified liver mitochondria (Rardin et al., 2013b). One possible explanation for this discrepancy could be the use of different antibodies in the two studies (Park et al., 2013; Rardin et al., 2013b). Nevertheless, despite their differences, these studies provide a comprehensive picture of the Sirt5-regulated succinylome in mammalian cells, a snapshot of which is shown in **Table 3** (Park et al., 2013; Rardin et al., 2013b).

REGULATION OF METABOLISM BY SUCCINYLATION AND Sirt5

Prominent targets of succinylation are the major metabolic processes, including the TCA cycle, the pathways breaking down

Table 3 | Protein succinylation, regulation by Sirt5 and functionally important sites.

Metabolic pathway	Substrate protein	K-Su sites targeted for desuccinylation by Sirt5		K-Su site associated with regulation	References	Site found both as K-Su and K-Ac
		MEFs (Park et al., 2013)	MLM (Rardin et al., 2013b)	-	-	Park et al., 2013; Rardin et al., 2013a,b
Glycolysis	Pkm	K498				N
Fatty acid oxidation	Schad	K81				N
	Hadha		K60			Y
			K166			N
			K644			Y
		K644	K644			N
	Eci2		K49			Y
			K52			Y
		K60				Y
	Lcad		K322			Y
	Acaa2		K209			Y
			K214			Y
PDHc	Pdha	K313		Su probably activates	Park et al., 2013	Y
TCA cycle	Cs	K450	K450			N
	Sdha	K179	K179	Su probably activates	Park et al., 2013	Y
	Mdh2		K239			Y
ETC	Ndufb11		K43			Y
OXPHOS	Atp5a1	K161				N
ATP handling			K427			Y
	Atp5b		K522			Y
	Atp5h	K78				N
	Atp5o		K73			Y
			K90			Y
	Ant1	K147				N
	Ant2		K43			Y
			K47			N
		K147				Y
Aminoacid catabolism	Glud1		K90			Y
Urea cycle			K480			Y
Ketogenesis	Otc		K221			Y
			K292			Y
	Cps1		K603	Su at K1291 probably inhibits	Du et al., 2011	Y
			K1291			Y
			K1486			Y
	Acat1		K260			Y
		K265	K265			N
	Hmgcs2		K83	K83E and K310E mutants inhibit	Rardin et al., 2013b	Y
			K310			Y
	Hmgcl		K48			Y

(Continued)

Table 3 | Continued

Metabolic pathway	Substrate protein	K-Su sites targeted for desuccinylation by Sirt5		K-Su site associated with regulation	References	Site found both as K-Su and K-Ac
		MEFs (Park et al., 2013)	**MLM (Rardin et al., 2013b)**	-	-	**Park et al., 2013; Rardin et al., 2013a,b**
Redox balance and sensing	Idh2		K360			Y
		K413				Y
	Aco2		K144			Y
		K628				N
	Sod1	Not found	(n/a)	K123E mutant inhibits	Lin et al., 2013	N
	Sod2	K122				N
	Catalase		K522			N
	Prdx1	K136	(n/a)			Y
	Me2	K224	(n/a)			N
Mitochondrial chaperones	Hspa9		K138			Y
		K600				Y
	Hspd1		K236			Y
	Hspe1		K36			N
			K56			Y
	CypD		K189			N

MEFs, mouse embryo fibroblasts; MLM, mouse liver mitochondria; n/a, not applicable (e.g., sod1 is a cytosolic/nuclear protein). All protein names can be referenced to Uniprot for more details. K-to-E are used as succinyl-mimetic mutants to probe whether the residue's succinylation alters enzymatic activity. The table shows a short selection of sites and Sirt5 substrates. For further information the reader is referred to the indicated studies.

amino acids and fatty acids, and the protein complexes responsible for energy transfer and ATP synthesis. This tropism toward metabolic pathways should not be surprising however, since the majority of studies performed so far focus on liver, or liver-derived material (Du et al., 2011; Fritz et al., 2013; Park et al., 2013; Rardin et al., 2013b; Weinert et al., 2013c). Because succinylation is so widespread, it is challenging to precisely pinpoint the sites that are key to regulation. To this end, defining the Sirt5-regulated succinylome simplifies this quest (**Table 3**). However, even for this sub-fraction of the succinylome, we lack the mechanistic insights of how succinylation affects function. From a few select examples, it appears that Sirt5-mediated desuccinylation can either enhance, or repress enzymatic activity. According to one study, Sirt5 desuccinylates subunits of the pyruvate dehydrogenase complex (PDHc) and this reduces PDH's activity (Park et al., 2013). A similar mode of regulation is suggested for respiratory complex II (SDH) (Park et al., 2013). If Sirt5 proves to have inhibitory effects on enzymes, this would distinguish it from Sirt3 (activates enzymes by deacetylation). According to a second study however, Sirt5 has activating effects, as it desuccinylates Hmgcs2 to increase hepatic ketogenesis (Rardin et al., 2013b). Sirt5 is also suggested to desuccinylate and activate enzymes regulating fatty acid oxidation in the liver and skeletal muscle (Rardin et al., 2013b). Therefore, though it may be early to suggest that Sirt5-mediated desuccinylation generally opposes actions of Sirt3-mediated deacetylation, it does suggest that lysines targeted by both PTMs may have important regulatory roles (**Table 3**).

The current evidence suggests that Sirt5 down-regulates glycolysis and the TCA cycle by de-succinylating and inhibiting PDH and SDH complexes (Park et al., 2013), while in separate studies Sirt5-mediated de-succinylation up-regulates fatty acid oxidation, ketogenesis and the urea cycle by activating Hmgcs2, Cps1 and other hepatic enzymes (Du et al., 2011; Rardin et al., 2013b). In the former study, inhibition by Sirt5 was determined in MEFs and extended in HEK cells (Park et al., 2013) while, in the latter, enzyme activation was shown in mouse liver and skeletal muscle and extended to primary hepatocytes, MEFs and HEK cells (Rardin et al., 2013b). Notwithstanding the differences between experimental models and methods, Sirt5 activity might act to influence mitochondrial substrate selectivity, perhaps shunning pyruvate in favor of fatty acids.

Differences are observed regarding the birth ratios of the deficient animals; one strain yields Sirt5$^{-/-}$ animals at normal Mendelian ratios while a second strain yields Sirt5$^{-/-}$ animals at lower than normal ratios (Lombard et al., 2007; Yu et al., 2013). One strain of Sirt5$^{-/-}$ mice is metabolically impaired, exhibiting reduced ketogenesis and fatty acid oxidation during fasting (Rardin et al., 2013b). A second strain however, appears to be

metabolically tolerant exhibiting mild improvements in insulin sensitivity in lean and obese states (Yu et al., 2013). Nevertheless, both strains exhibit elevated blood ammonia levels (hyperammonemia) in the fasted state (Nakagawa et al., 2009; Yu et al., 2013). Although all of the above observations are not mutually exclusive, they highlight potential complexities in the regulation of metabolism by Sirt5.

PROTEIN SUCCINYLATION AND Sirt5 IN THE HEART

These are early days, indeed, for the study of succinylation in the heart. Very little data are available exploring the magnitude of protein succinylation, its implications in protein function, and its impact on cardiac function. The baseline phenotype of Sirt5$^{-/-}$ animals does not reveal significant cardiac abnormalities and several parameters related to heart function, including blood pressure, heart rate, and endurance exercise tolerance are normal (Yu et al., 2013). Nevertheless, mitochondria isolated from Sirt5$^{-/-}$ hearts exhibit considerable increases in global protein succinylation (Tan et al., 2014). At the cellular level, limited data suggest a cytoprotective role for Sirt5 in cardiac myocytes. According to one study, Sirt5 opposes H_2O_2–induced death in H9c2 cells and NRVMs (Liu et al., 2013). Mechanistically, the enzymatic activity of Sirt5 appears to be important for the cytoprotective effect and Sirt5 is demonstrated to physically interact with the anti-apoptotic protein Bcl-xL, but not Bcl-2 (Liu et al., 2013). Another study found that Sirt5 protein is upregulated in the heart during intermittent hypoxia (Zhu et al., 2012), a regimen thought to stimulate cardiac tolerance to stress. Collectively, the findings suggest potential roles of Sirt5 in stress-induced cardioprotection, but the limited number of studies and the scarcity of data on cardiac protein succinylation warrant additional investigations before firm conclusions can be made.

LYSINE MODIFICATION BY OTHER DICARBOXYLATES: MALONYLATION AND GLUTARYLATION

MALONYLATION

Malonyl-CoA is a metabolite important in fatty acid biosynthesis, derived from the carboxylation of acetyl-CoA in the cytoplasm and mitochondria (**Figure 2**). Lysine malonylation (K-Ma) was identified as a new PTM that induces a mass shift of 86 kDa in the spectra of modified peptides (Du et al., 2011; Peng et al., 2011) and, similar to succinylation, malonylation of lysines adds negative charge to the modified residue (see also **Figures 2**, **3**). Moreover, like K-Su, K-Ma is also a substrate for Sirt5 (Du et al., 2011; Peng et al., 2011). Consistently, Sirt5$^{-/-}$ mice exhibit enhanced protein malonylation compared to wild-type mice (Peng et al., 2011). Protein malonylation is detected in *E.coli*, in numerous mammalian cells and tissues; targets include mitochondrial and extramitochondrial proteins (Du et al., 2011; Peng et al., 2011; Xie et al., 2012). Using immunoenrichment followed by LC-MS/MS, 17 malonylated proteins are identified in HeLa cells (Peng et al., 2011). Another study utilizing a membrane-permeable malonate analog, labeled, and identified 375 proteins as candidates for malonylation in HeLa cells (Bao et al., 2013). Interestingly, only a small fraction of these proteins (9%) were known mitochondrial proteins (Bao et al., 2013). This may indicate that malonylation occurs predominantly on extramitochondrial locations, or perhaps that the method is less suitable for targeting of mitochondria. Isotopic labeling with sodium malonate in HeLa cells indicates that protein malonylation occurs within 24 h (mitochondrial proteins Hspa9 and Hspe1 were identified) (Peng et al., 2011). *In vivo*, reductions in protein malonylation (attributable to sirtuin activity) are evident within 2 h in HeLa cells (Bao et al., 2013). As mentioned previously, the dynamics of succinylation depend on the availability of succinyl-CoA (Weinert et al., 2013c). The case may be similar for malonylation as well, where exposure to sodium malonate presumably increases intracellular malonyl-CoA and potentiates protein malonylation (Peng et al., 2011). Finally, the finding that malonylation is under the negative regulation of Sirt5, cautions that current or future phenotypes identified after loss-/gain-of function approaches of Sirt5 in cells and animals, can be attributable to excess protein malonylation (together with succinylation, discussed above and glutarylation, discussed below). To this point, no direct evidence is available that describes the effect of malonylation on substrate function and its role in mammalian physiology.

GLUTARYLATION

Protein glutarylation is the latest addition to the growing list of lysine acylations identified (Tan et al., 2014). Glutaryl-CoA is produced in mitochondria as a result of the catabolism of tryptophan and lysine (Borsook et al., 1948a,b; Gholson et al., 1962). Glutaryl-CoA contains a negatively charged carboxyl group and is structurally similar to malonyl-CoA and succinyl-CoA. Compared to malonylation and succinylation (identified by a shift of 86 and 100 da respectively), glutarylation causes a shift of 114 Da to the spectra of peptides bearing a modified lysine (Tan et al., 2014). Kinetic and endpoint assays using numerous substrates indicate that Sirt5 has high enzymatic activity for deglutarylation (Tan et al., 2014). The interaction of Sirt5 with malonyl-, succinyl-, and glutaryl-lysines is favored by ionic interactions between the carboxyl group of the substrate, residues forming the substrate binding pocket of Sirt5, as well as the co-substrate, NAD^+. Moreover, glutaryl-lysine is probably the largest substrate Sirt5 can accommodate, since adipoyl-lysine (which is longer than glutaryl-lysine by one methylene group) is not cleaved by Sirt5 under *in vitro* conditions (Tan et al., 2014).

Conditions favoring protein hyperglutarylation in cells and tissues include: (i) exposure to high concentrations of sodium glutarate, (ii) diet rich in tryptophan, (iii) deficiency of the enzyme catabolizing glutaryl-CoA (glutaryl-CoA dehydrogenase, Gcdh), (iv) prolonged fasting (i.e., for 48 h) and (v) gene deficiency of Sirt5 (Tan et al., 2014). In Sirt5$^{-/-}$ liver lysate, 191 glutarylated proteins are identified by immunoenrichment and HPLC-MS/MS. The majority of them (148), are known mitochondrial proteins which would be consistent with mitochondria as the site of glutaryl-CoA synthesis and Sirt5 as the primary mitochondrial deglutarylase. Glutarylation accumulates on proteins over the course of several hours (within 6 h in enzyme-free reactions and up to 24 h in cells). By contrast, deglutarylation is completed as early as 30 min in the presence of Sirt5 (Tan et al., 2014).

In sum, significant parallels exist in the dynamics and regulation of glutarylation and succinylation (and to a lesser extent, malonylation) illustrating that the role of Sirt5 is more complex than once thought. Among various tissues, protein glutarylation is also detectable in heart mitochondria and it remains to be determined whether this PTM exerts important roles in cardiac function and dysfunction. A first step in addressing these issues would be to determine the baseline levels of glutaryl-CoA in the heart and test whether these fluctuate in response to physiologic and pathologic stimuli. Serving as a genetic model of glutaryl-CoA elevation, $Gcdh^{-/-}$ mice exhibit underlying protein hyperglutarylation (Tan et al., 2014). These mice were found to have fully functional mitochondrial respiratory complexes and normal cardiac histology (Koeller et al., 2002; Sauer et al., 2005). Reassessing the phenotype of these mice in light of protein hyperglutarylation might help identifying important regulatory functions of this PTM in cardiac function.

STEPS TOWARD CHARACTERIZING PROPIONYLATION, BUTYRYLATION, AND CROTONYLATION

Found in the form of thioesters with coenzyme A, propionyl-, butyryl-, and crotonyl- groups are common intermediates in energy metabolism. The breakdown of odd- and even-chain fatty acyl-CoAs through mitochondrial β-oxidation is major source of propionyl-CoA and butyryl-CoA (**Figure 2**). Moreover, the unsaturated variant, crotonyl-CoA derives from butyryl-CoA or glutaryl-CoA through processing by dedicated mitochondrial dehydrogenases (Green et al., 1954; Lenich and Goodman, 1986). Similarly to acetyl-CoA, propionyl-, butyryl-, and crotonyl-CoA do not have charged groups, which distinguishes them from the dicarboxylate derivatives malonyl-, succinyl-, and glutaryl-CoA which have a negatively charged group. Acetyl-CoA, propionyl-CoA butyryl-CoA, likewise all share the same Gibbs free energy of thioester bond hydrolysis (−36 kJ/mol) (Thauer et al., 1977), and the extended family of acyl-CoA compounds may well exhibit similar reactivity toward lysine residues. Examples of lysine propionylation, butyrylation, and crotonylation (K-Pr, K-Bu, and K-Cr respectively) are identified in bacteria, yeast and mammalian cells. With a few exceptions, most studies focus on the roles these PTMs have on histone and chromatin regulation. Nevertheless, given the mitochondrial origin of propionyl-, butyryl-, and crotonyl-CoAs, it is anticipated that the repertoire of K-pr, K-bu, and K-cr will expand to include mitochondrial proteins as well. Steps toward that direction have already been made (Fritz et al., 2013; Pougovkina et al., 2014b).

PROPIONYLATION

K-Pr results in a shift of 56 Da on the mass of peptides bearing the modification (for comparison, K-Ac causes a shift of 42 da). In the prokaryote *S.enterica*, K-Pr is identified on the enzyme propionyl-CoA synthetase (PrpE) (Garrity et al., 2007). In mammalian cells, K-Pr is found on nuclear proteins p300, CBP, histones H3 and H4 and p53 (Chen et al., 2007; Cheng et al., 2009; Liu et al., 2009). More recently, a total of 42 propionylated proteins were identified in mouse liver, of which 23 came from the mitochondrial fraction (e.g., Acaa2, Cps1, Hmgcs2, catalase) (Fritz et al., 2013). Propionylation likely influences protein function, but specific examples are currently limited. In the case of *S.enterica*, propionylation is suggested to reduce PrpE's enzymatic activity (Garrity et al., 2007). Moreover, high levels of propionyl-CoA inhibit PDHc, αKGDHc and respiratory complex III, although lysine propionylation was not directly tested (Schwab et al., 2006).

A number of studies support that K-Pr is positively regulated by propionyltransferases. The Gcn5-related prokaryotic acetyltransferases Pat and AcuA propionylate PrpE in the presence of propionyl-CoA *in vitro* (Garrity et al., 2007). Moreover, eukaryotic acetyltransferases p300 and Cbp propionylate histones and p53 *in vitro* and *in vivo* (Chen et al., 2007; Cheng et al., 2009; Liu et al., 2009). *In vitro* evidence also shows Pcaf to operate as a propionyltransferase for histone H3 (Leemhuis et al., 2008), although Pcaf was found to be inefficient for protein propionylation in other studies (Chen et al., 2007; Cheng et al., 2009). Nuclear propionyltransferase targets include histone H3K14, H3K23, H4K12, and p53 K292. By comparison, in mitochondria, there is currently no direct evidence for an equivalent enzymatic activity. As suggested for other PTMs, propionyl-CoA levels could be a dominant force shaping the mitochondrial propionylome. Consistently, incubating liver mitochondrial lysates with increasing concentrations of propionyl-CoA elevates protein propionylation (Pougovkina et al., 2014b). Moreover, protein propionylation increases in liver mitochondria of mice exposed to ethanol for 3 or 6 weeks (Fritz et al., 2013) though whether this elevation is the result of propionyltransferase activation, depropionylase inhibition or changes in mitochondrial propionyl-CoA flux, remains to be determined. Regarding dynamics, enzymatic propionylation *in vitro* is completed within 30–120 min (Chen et al., 2007; Garrity et al., 2007; Leemhuis et al., 2008; Liu et al., 2009), whereas chemical propionylation of mitochondrial lysates progresses over the course of 3 h (Pougovkina et al., 2014b).

Negative regulation of protein propionylation by enzymes is a subject of ongoing research. The prokaryotic sirtuin homolog CobB (from the bacterium *S.enterica*) depropionylates PrpE *in vitro*, in a reaction that requires NAD^+ and produces O-Pr-ADPR (Garrity et al., 2007) (see **Figure 3** for similar reactions). PrpE is also depropionylated by another prokaryotic sirtuin homolog (Sir2, from the bacterium *T.maritima*) and by mammalian sirtuins Sirt2 and Sirt3 (Garrity et al., 2007). Measurable *in vitro* depropionylation of synthetic peptides (H3K14-Pr, H3K23-Pr, H3K9-Pr) was also reported for sirtuins Sirt1 and Sirt2, while Sirt3 does not appear as potent (Smith and Denu, 2007; Liu et al., 2009; Feldman et al., 2013). *In vivo*, Sirt1 opposes p53's propionylation in HEK293 cells (Cheng et al., 2009). In liver mitochondria, global protein propionylation is not significantly affected by Sirt3 deficiency (Fritz et al., 2013). Thus, the mitochondrial depropionylase remains elusive, since neither Sirt4 nor Sirt5 are efficient catalysts *in vitro* thus far (Garrity et al., 2007; Feldman et al., 2013).

Little is known regarding the stoichiometry of K-Pr. It is likely that the sites identified thus far represent those with the highest stoichiometry. For reference, K-Pr stoichiometry at H3K23 is estimated to be around 7% in a leukemia cell line (Liu et al., 2009). Moreover, K-Pr on nuclear proteins occurs rather infrequently compared to K-Ac (Cheng et al., 2009). Regarding co-occurrence

of acetylation and propionylation at the same K residue (i.e., K-Ac and K-Pr overlap), some information for nuclear proteins suggests that it exists. Moreover, considerable overlap is identified between K-Ac and K-Pr in liver mitochondria (Fritz et al., 2013). The role of lysine propionylation in human disease is only now beginning to be addressed. Deficiency in propionyl-CoA carboxylase (Pcc) in humans results in propionic aciduria, developmental and metabolic defects. Fibroblasts from these patients have protein hyperpropionylation likely resulting from propionyl-CoA accumulation (Pougovkina et al., 2014b). It remains unknown whether protein hyperpropionylation has a causative, or aggravating role in the progression of the disease. The advent of more comprehensive propionylomes will provide a better understanding of this PTM and shed light on its potential implications to cardiac disease.

BUTYRYLATION

Larger than propionyl-CoA by one methylene group, butyryl-CoA has similarities to propionyl-CoA in terms of its origin, ability to modify lysines and removal by putative de-butyrylases. The first examples of protein lysine butyrylation came by searching for novel PTMs in histone proteomics data. Following a series of validation steps, mass shifts of 70 da in modified peptides were assigned to lysine butyrylation (K-Bu) in histones H3, H4, and nuclear proteins p53, p300, and CBP (Chen et al., 2007; Cheng et al., 2009). In the case of histone H4, butyrylated lysines (e.g., H4K5, H4K8, H4K12, H4K31) were also found to be propionylated, attesting to a significant overlap between K-Bu and K-Pr (Chen et al., 2007). Among 5 HATs, only CBP and p300 can operate as butyryl-transferases *in vitro* and this activity is extended *in vivo* (Chen et al., 2007; Cheng et al., 2009). Owing to CBP and p300 activity, p53 is butyrylated at two positions, K373 and K382 in lung cancer cells (Cheng et al., 2009). Histone butyrylation is also detected in the mouse brain (e.g., H2AK95 and H3K115) (Tweedie-Cullen et al., 2012). There, the stoichiometry of K-Bu at H3K115 was found to be 31%, implying a critical role of butyrylation at this histone site.

In mitochondria, K-Bu is readily detectable by a butyryl-lysine-specific antibody (Fritz et al., 2013). This antibody can be useful in enriching for butyrylated peptides from mitochondrial proteins and identifying them with mass spectrometry. Sirt3 deficiency does not dramatically alter butyrylation in MLM suggesting that Sirt3 is not a major regulator (Fritz et al., 2013), in apparent agreement with *in vitro* studies showing that Sirt3 has low de-butyrylation activity (Smith and Denu, 2007; Feldman et al., 2013). While it is unclear what sirtuin opposes mitochondrial butyrylation (Sirt4 and Sirt5 have no debutyrylase activity; Feldman et al., 2013), emerging evidence suggests that butyryl-CoA flux is a major determinant of butyrylation rates. Genetic deficiency in butyryl-CoA dehydrogenase substantially increases protein butyrylation in liver and this is attributed to aberrant accumulation of hepatic butyryl-CoA (Pougovkina et al., 2014b). Since butyrylation is upregulated, this genetic model could be useful in deciphering the regulatory enzymes opposing protein hyperbutyrylation and also help identify important substrates undergoing butyrylation in various tissues including liver and heart.

CROTONYLATION

The identification crotonylation as a novel lysine PTM (68 Da), stems directly from efforts to increase histone protein coverage by derivtizing peptides with propionic anhydride prior to HPLC-MS/MS (Tan et al., 2011). Comparison of the HPLC-MS/MS spectra and elution properties of synthetic and *in vivo* peptides, validated K-Cr as a naturally occurring PTM. This was further substantiated by identifying K-Cr sites after isotopic labeling with D4-crotonate in cells, and with detection of modified proteins using anti-crotonyl-lysine specific antibody (Tan et al., 2011). Examples of K-Cr sites on histones include H2AK36, H2BK5, H3K23, and H4K12. A separate study identifies K-Cr on brain histones, including H2AK95, H2BK108, H3K122, and H4K91, with stoichiometries ranging from 1–3% (Tweedie-Cullen et al., 2012). In contrast to other lysine acylations, K-Cr is structurally more rigid, as the double bond restricts rotation of the carbon atoms. Whether this rigidity has unique implications in histone function remains unknown. One interesting observation is that histone crotonylation is very frequent in gene enhancers and it prevents chromosome inactivation of differentiating sperm cells (Tan et al., 2011).

While the above study laid the framework, questions still remain about the regulation of K-Cr and its impact on non-histone and perhaps mitochondrial proteins. The dynamics of K-Cr are likely governed by crotonyl-CoA levels. Altering the flux of crotonyl-CoA (a metabolic intermediate in fatty acid and amino acid catabolism) could directly affect global mitochondrial protein crotonylation. Studies are also designed to identify enzymatic activities promoting or opposing crotonylation. In contrast to what has been shown for propionylation and butyrylation, the HATs p300 and CBP do not promote crotonylation (Tan et al., 2011). Furthermore, none of the 11 histone deacetylases (HDACs) can decrotonylate efficiently (Tan et al., 2011). A screen of the sirtuins for decrotonylation activity using a synthetic peptide (H4K12-Cr) found Sirt1 to be somewhat active, while Sirt2, 3, 5 and 6 had negligible activity (Madsen and Olsen, 2012). A separate study using a different substrate (H3K9-Cr) found measurable decrotonylase activities in Sirt1 and Sirt2 but not in Sirt3-Sirt6 (Feldman et al., 2013). In aggregate, sirtuins Sirt3-Sirt5 are unlikely to be decrotonylases, so a dedicated mitochondrial decrotonylase remains to be discovered. An alternative possibility is that the crotonyl moiety is modified *in situ* to become the substrate of one of the mitochondrial sirtuins (e.g., conversion of K-Cr to K-Ac through a series of enzymatic steps and final deacetylation by Sirt3).

The importance of K-Cr to mitochondrial protein activity has yet to be established. A genetic model of protein hypercrotonylation would be valuable in this regard, just as Gcdh and Bcdh-null models have provided insights into the roles hyperglutarylation and hyperbutyrylation respectively (Pougovkina et al., 2014b; Tan et al., 2014). Protein hypercrotonylation might be possible by inactivating crotonase, a multisubunit enzyme converting crotonyl-CoA to 3-hydroxybutyryl-CoA (Engel et al., 1996). One limitation could be that the levels of enoyl-CoA intermediates other than crotonyl-CoA might be affected, since crotonase has broad specificity (Waterson and Hill, 1972; Schulz, 1974; Fong and Schulz, 1977). More investigation is warranted for this newly

discovered PTM and its potential implications in mitochondrial protein regulation in heart and other tissues.

CONCLUSIONS

Once exclusively the purview of histone research, the study of lysine PTMs has undergone a profound maturation. Mitochondria are epicenters for an evolving class of lysine PTMs including succinylation, malonylation, and glutarylation. The result has been a data deluge. And so begins the daunting task of piecing it all together and sorting the regulatory PTMs from the epiphenomena of metabolic stress (Beltrao et al., 2012). And with these discoveries comes the realization that newer tools may be required to assess the causal relationship between PTM and protein function. For instance, until now, mutation of K to Q has served as an acceptable mimic of the charge neutralization of acetylation, while K to E mutation has served to mimic the negative charge of succinylation. Are these mutations equally suitable for the study of propionylation, malonylation, and glutarylation, whose size differ? Promisingly, Neumann et al. have developed a method to introduce site-specific acetylated lysine residues in recombinant proteins expressed in *E.coli* (Neumann et al., 2008). If the method can be honed to incorporate other modified lysines, it will be possible to test their effect on function and the extent to which K to Q/E mutation may serve as a valid proxy in mammalian cell culture.

The new lysine modifications join the pantheon of metabogenic PTMs, including O-GlcNacylation and palmitoylation among others. And while great strides have been made to understand how specific nutrients act as ligands to activate transcriptional programs to modulate metabolism, it is also clear now that metabolism, itself, leaves its mark on the proteome in a manner that ultimately affects the activity of enzymes. This increased complexity and nuanced regulation may afford new opportunities for therapeutic intervention in disease and the mitochondrial sirtuins promise to be prime targets. Already, emerging evidence shows that increasing the availability of NAD^+ (e.g., through dietary supplementation of its biosynthetic intermediates) can boost sirtuin activity and improve mitochondrial function, conferring salutary effects in diabetes and aging (Yoshino et al., 2011; Canto et al., 2012; Mouchiroud et al., 2013). As we delve deeper into the role of newly-found lysine modifications in heart disease, it may also be desirable to develop chemical screens for Sirt3 and Sirt5-specific modulators.

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Necroptosis: is there a role for mitochondria?

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Once thought to be a random process of cell death, necrosis can proceed via a defined molecular mechanism and is integral to physiological and pathological states. In particular a form of necrosis called necroptosis has been the subject of intense investigation. Necroptosis is initiated by tumor necrosis factor-α (TNFα), which leads to the activation of the kinase receptor-interacting protein 1 (RIP1). RIP1 then binds with and activates RIP3 to form the necrosome. RIP3 in turn interacts with and activates the pseudokinase mixed lineage kinase domain-like (MLKL). This complex has then been proposed to induce necrotic death via the induction of mitochondrial dysfunction, with a variety of mechanisms being put forth including: production of mitochondrial reactive oxygen species (ROS), activation of the mitochondrial phosphatase PGAM5, or induction of mitochondrial permeability transition (MPT). However, recent evidence suggests that none of these are involved in necroptosis, and that mitochondria may in fact be dispensable for this process. Therefore, the purpose of this perspective is to discuss the current understanding of necroptosis, and more specifically, what role if any do mitochondria play in this mechanism of cell death.

Keywords: necrosis, necroptosis, mitochondria, reactive oxygen species, TNFα

INTRODUCTION

Cells die for a variety of reasons and under myriad circumstances. Cells are thought to undergo three major forms of regulated cell death: apoptosis, autophagy, and necrosis. In contrast to apoptosis and autophagy, necrosis was once thought of as a random uncontrolled form of cell death. However, it has been established that necrosis can proceed via a controlled and varied series of events. The most studied and defined molecular necrotic pathway is traditionally mediated by signaling by the tumor necrosis factor-α (TNFα) receptor (TNFR) through RIPs 1 and 3 and subsequently the pseudokinase MLKL and is termed necroptosis (Christofferson et al., 2014; Galluzzi et al., 2014; Vanden Berghe et al., 2014).

From its earliest description, execution of necroptosis has been thought to involve ROS and mitochondria (Van Herreweghe et al., 2010). This assumption has been perpetuated by countless studies in the nearly 10 years since the first use of the term necroptosis. Recent evidence casts doubt on the idea that signaling via the necrosome requires ROS and mitochondria. In fact, cells are able to undergo necroptosis in the near absence of mitochondria and ROS is dispensable for this event (Tait et al., 2013).

While the proximal molecular componentry of the necroptotic pathway is known, its downstream signaling, until recently, has been poorly understood. Although mitochondria in a variety of guises have been proposed to be mediators of necroptosis, recent data has suggested that they may not in fact be necessary for the execution of the necroptotic program. This perspective will therefore summarize the current understanding of necroptosis signaling and discuss whether mitochondria have any role to play in this form of programmed necrosis.

Abbreviations: CYLD, cylindromatosis; CypD, cyclophilin-D; Drp1, dynamin-related protein 1; GLUD1, glutamate dehydrogenase 1; MLKL, mixed lineage kinase domain-like; MPT, mitochondrial permeability transition; RIP, receptor interacting protein; ROS, reactive oxygen species, TNFα, tumor necrosis factor-α, TNFR, tumor necrosis factor-α receptor; TRADD, TNFR1-associated death domain protein.

PROGRAMMED NECROSIS AND THE NECROSOME

The pro-inflammatory cytokine TNFα plays an important role in inducing cell death (Van Herreweghe et al., 2010; Vanden Berghe et al., 2014). In response to TNFα stimulation, TNFR recruits proteins to the plasma membrane (Van Herreweghe et al., 2010; Christofferson et al., 2014; Vanden Berghe et al., 2014). When TNFR trimerizes in response to ligand binding, TNFR1-associated death domain protein (TRADD) is recruited to the cytoplasmic domain of this receptor (Hsu et al., 1995). The TNFR/TRADD complex then recruits RIP1 and TNFR-associated factor 2/5 (TRAF2/5) to the plasma membrane (Hsu et al., 1996a,b; Tada et al., 2001; Ermolaeva et al., 2008). Next, cellular inhibitor of apoptosis proteins (cIAP1/cIAP2) are recruited to the TNFR which are involved in inhibition of RIP1 and RIP3 signaling (McComb et al., 2012). Together with the TNFR, this assemblage of proteins is known as complex I, and is responsible for signaling a variety of cell behaviors including proliferation, survival and NF-κB signaling (Christofferson et al., 2014). Complex I can transition from a membrane associated protein assembly to the cytosol. This cytosolic collection of proteins is known as complex IIa and in place of TNFR from complex I contains caspase 8 and adaptor protein FADD (Christofferson et al.,

2014; Vanden Berghe et al., 2014). Formation of complex IIa can yield two cell fates, apoptosis or necrosis, and when caspase 8 is inhibited, necrosis prevails.

Through the interaction of RIP1 and RIP3 necroptotic signaling is induced (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). RIP1 and RIP3 comprise the necrosome, on which this perspective will be focused. Post-translational modifications of RIP1 and RIP3 are crucial steps for the initiation of necrosome formation and signaling (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). Initially, cylindromatosis (CYLD) a deubiquinase has been shown to deubiquinate RIP1 after complex I dissociates from the TNFR and facilitate necrosome signaling (Moquin et al., 2013). This deubiquitination activates RIP1 so that it can bind to and phosphorylate RIP3. This activates RIP3, whose kinase activity is required for necroptotic signaling (Cho et al., 2009; He et al., 2009; Newton et al., 2014). Recent experiments have begun to elucidate signaling events in programmed necrosis downstream of the necrosome. Specifically, RIP3 dependent phosphorylation of the pseudokinase mixed lineage kinase domain-like protein (MLKL) has been shown to be essential for RIP1/RIP3-dependent necroptosis (Sun et al., 2012; Zhao et al., 2012; Murphy et al., 2013). However, at this point the sequence of events downstream of RIP1/RIP3/MLKL that ultimately lead to cell rupture becomes muddled. Contradictory experimental results point toward two different mechanisms of necroptosis, either dependent on the mitochondria through a variety of mechanisms, or independent of these events. The remainder of this perspective will focus on the evidence supporting these two divergent hypotheses.

THE ROLE OF MITOCHONDRIA IN NECROPTOSIS: EVIDENCE FOR

Necrosomal activation is known to induce programmed necrosis, however new results call into question the mechanism by which this process progresses. Classically, necrosomal signaling was thought to involve ROS generation from the mitochondria in the execution of cell death. An association between necrosome signaling and ROS generation is illustrated in many studies. The complex II and necrosome proteins RIP, TRAFF, and FADD have been shown to be critical for the accumulation of ROS in TNFα signaling (Lin et al., 2004). In this exploration of necroptotic signaling, mouse embryonic fibroblasts (MEFs) (WT, RIP1$^{-/-}$, TRAFF$^{-/-}$ and FADD$^{-/-}$) were exposed to TNFα, and ROS was measured by dichlorofluorescin diacetate (DCFDA). From their data, it was proposed that necroptotic signaling proceeds through the TNFα-induced accumulation of ROS. The necessity of ROS for this process was confirmed via the use of the antioxidant butylated hydroxyanisole, which blocked ROS accumulation and the comsensurate cell death. TNFα mediated ROS generation has been shown to be dependent on RIP1 and mitochondrially derived in L929 cells (Vanlangenakker et al., 2011). This was determined by inhibiting cytoplasmic ROS generation by siRNA mediated knockdown of NADPH oxidase components, which did not affect TNFα mediated cell death. However, with suppression of complex I of the respiratory chain, TNFα mediated cell death was attenuated. In support of this finding, mitochondrial, but not cytosolic ROS is critical in mediating TNFα induced cell death in L929 and RAW 264.7 cells (Ardestani et al., 2013). These data confirmed the results of Vanlangenakker in L929 cells and extended this finding to a different cell type, a mouse monocyte line. Studies have indicated that complex I of the electron transport chain is responsible for the ROS production seen during TNFα-induced necroptosis (Schulze-Osthoff et al., 1992; Goossens et al., 1999). In both of these studies, ROS generation was measured in L929 cells. This coupled with the fact that RIP1, RIP3, and/or MLKL have all been reported to translocate to the mitochondrial fraction upon stimulation in a variety of cell types (Temkin et al., 2006; Zhang et al., 2009; Davis et al., 2010; Wang et al., 2012), indicated that ROS production could indeed be a key step in the execution of the necroptotic process. In addition to showing mitochondrial translocation of RIP3 to the mitochondria in MEFs, Davis et al. were able to inhibit necrotic cell death in endothelial cells with the mitochondrial antioxidant MnSOD (Davis et al., 2010). From the current data, it is clear that certain cell types, such as L929 cells, are heavily represented in the study of necroptosis. However, it is important to note that mitochondrial ROS is implicated in TNFα induced necrosis by several groups using other cell types (MEFs, endothelial cells and RAW 264.7 cells).

Another potential mitochondrial mediator of necroptosis and one that may provide a link to ROS production is the mitochondrial permeability transition (MPT) pore. The MPT pore is a large, non-specific channel that spans the inner mitochondrial membrane. Opening of the MPT pore leads to a loss of the mitochondrial transmembrane potential and failure of oxidative phosphorylation, production of ROS, and ultimately swelling and rupture of the organelle (Baines, 2010). Genetic experiments where a critical regulator of the MPT pore, cyclophilin-D (CypD), was knocked out revealed a role for the MPT pore primarily in necrotic cell death as opposed to apoptosis (Baines et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). Whether the MPT pore couples to necroptotic signaling has been the subject of considerable study. TNFα-induced necroptosis was found to be partially attenuated by the loss of CypD in mouse embryonic fibroblasts (He et al., 2009). Similarly, TNFα-induced zebrafish macrophage ROS production and necrosis was blocked by the CypD inhibitor alisporivir (Roca and Ramakrishnan, 2013). In the myocardium, protection against ischemia/reperfusion by the RIP1 inhibitor necrostatin was not additive to that conferred by CypD ablation, also suggesting that the 2 components were part of the same genetic pathway (Lim et al., 2007).

There is also evidence the other mitochondrial functions/components may play a role in TNFα-induced necroptosis. For example, Zhang et al. (2009) found that RIP3 could translocate to the mitochondria where it interacted with the mitochondrial protein glutamate dehydrogenase 1 (GLUD1). Consistent with this they demonstrated that knockdown of GLUD1 could at least partially block TNFα-induced ROS production as measured by DCFDA staining and necrosis in NIH 3T3 cells. Bcl2 family proteins may also play a role in necroptosis. Specifically, Hitomi et al. (2008) identified the Bcl2 protein Bmf in a proteomic screen for potential mediators of TNFα-induced necroptosis, and demonstrated that at least in L929 cells depletion of Bmf could attenuate the necrotic response to TNFα. That being said, how

exactly Bmf is functioning at the mitochondria and whether this is a conserved mechanism in other cell types has yet to be tested.

Finally, the interaction of RIP3 with MLKL has been reported to induce translocation of the RIP1/RIP3/MLKL complex to the mitochondrial membrane as TNFα induced necroptosis results in enriched levels of RIP1/RIP3/MLKL in the mitochondrial associated membrane fraction of cells, i.e., the contact sites between the outer mitochondrial membrane and the ER membrane (Chen et al., 2013). Upon translocation to the mitochondria, the necrosome interacts with and activates the mitochondrial phosphatase PGAM5 (Wang et al., 2012). Signaling of the necrosome through PGAM5 was then shown to result in mitochondrial fragmentation in a Dynamin-related protein 1 (Drp1) manner (Wang et al., 2012). These data were obtained in HeLa cells, and indicated a direct interaction between RIP1/RIP3 and PGAM5 as determined by immunoprecipitation. siRNA mediated knockdown of Drp1 and inhibition of Drp1 with mdivi-1 were able to inhibit TNF mediated necroptosis in both HeLa and HT-29 cells. In further support of this, Drp1 depletion decreases death in NRK-52E (rat renal tubule epithelial cells) treated with TNFα (Zhang et al., 2013).

THE ROLE OF MITOCHONDRIA IN NECROPTOSIS: EVIDENCE AGAINST

The studies described above all suggested that in one form or another, mitochondria played a pivotal role in the execution of the necroptotic program. However, several recent studies have questioned the role of each of the mitochondrial facets in necroptosis and suggest that mitochondria maybe dispensable for this process. For example, many early studies proposing a role for mitochondrial ROS merely show a circumstantial relationship between ROS, mitochondria and cell death. In their description of RIP3 as a component of the necrosome, He et al. called into question the necessity for ROS in necrosome signaling (He et al., 2009). Their results indicate that the requirement of ROS in necrosome mediated cell death may be cell type specific, where ROS scavenging protects L929 but not HT-29 cells from TNFα-induced necrotic cell death. Macrophage and Jurkat cell necrosis appears to be ROS-independent (Moquin and Chan, 2010), indicating that ROS are not absolutely required for necroptosis. In confirmation of this, the human monocyte derived THP-1 cell line shows an increase in ROS in response to TNF treatment, but treatment with a variety of antioxidants (ascorbic acid, glutathione, and *N*-acetly-cystein) does not attenuate death (Temkin et al., 2006).

Whether the MPT pore plays a role in necroptosis has also been questioned. Specifically, the embryonic lethality caused by caspase-8 deletion is due to RIP3-dependent necroptosis (Kaiser et al., 2011), and cannot be rescued by CypD ablation (Tait et al., 2013). Similarly, necroptosis in caspase-8 deficient macrophages could be blocked by depletion of RIP1 and RIP3 but not by depletion of CypD (Ch'en et al., 2011). Finally, a recent paper examining ischemia/reperfusion injury in the kidney found that ablation of RIP3 and CypD was protective but that double knockout mice exhibited even great protection (Linkermann et al., 2013), suggesting that the necrosome and the MPT pore are distinct pathways. This is opposite to that seen in the heart and raises the question of whether there are tissue-specific differences in the functional coupling of the MPT pore to RIP1-dependent signaling. Moreover, although CypD is a major regulator of the pore, it is not the pore itself and it maybe that necroptotic signaling can bypass CypD and directly activate the pore; in this scenario loss of CypD would not be expected to offer protection against caspase-8 deletion. Both of these issues need to be examined further.

The role of the mitochondrial PGAM5-Drp1 axis has also been questioned by several recent studies. Silencing of PGAM5 was found to have no effect on necroptosis induced by TNFα or RIP3 dimerization in a variety of cell lines (Murphy et al., 2013; Tait et al., 2013; Remijsen et al., 2014). Murphy et al. found that knocking down PGAM5 with shRNA in MEFs did not attenuate the response of MEFs to TNFα induced necrosis. Similar data were obtained by Remijsen et al. using a siRNA mediated knockdown of PGAM5 which did not block TNF induced necrosis in L929 cells. Similar results have been obtained when Drp1 was either silenced or knocked out (Moujalled et al., 2014; Remijsen et al., 2014). Again, in these experiments, Remijsen et al. utilized L929 cells. Moujalled et al. used DRP1$^{-/-}$ MEFs to show that cells can undergo necroptosis independently of DRP1. We ourselves have also found that PGAM5 and Drp1 are not necessary for TNFα-induced necroptosis to occur in both MEFs and HT-29 cells (unpublished data). Perhaps the most elegant study indicating that mitochondria are entirely dispensable for necroptosis was a recent paper by Doug Green's group (Tait et al., 2013). Here they depleted SVEC and 3T3 cells of mitochondria via the induction of mitophagy by the uncoupler carbonylcyanide m-chlorophenylhydrazone (CCCP). They demonstrated that although TNFα-induced ROS was lost in the mitochondrially-deficient cells, necroptosis was still very much functional, whether it was induced by TNFα or more directly by RIP3 dimerization. However, this study was not without limitations since about 20% of cells studied still contained some mitochondria. Thus mitochondria were not completely eliminated and could still therefore have contributed, at least partially, to the necroptotic response. In future studies, it would be of value to sort cells that contain and do not contain mitochondria following induction of necroptosis to further confirm that this process is mitochondria-independent.

Consistent with these findings, very recent studies have emerged indicating that MLKL may bypass the mitochondria altogether, instead translocating to the plasma membrane upon homo-oligermerization (Cai et al., 2014; Chen et al., 2014). Cai et al. utilized HEK293 cells to initially show that MLKL oligomerizes upon induction of necrosis. This oligomerization was confirmed under more physiologically relevant conditions by inducing necroptosis in HT-29, Jurkat, U937, MEF, and J774A.1 cells, indicating that this process occurs in multiple cell types. To measure translocation to the plasma membrane, HEK293 and HT-29 cells were transfected with a fluorescent MLKL construct. These results were confirmed biochemically by isolating cell surface proteins from HT-29, MEF and J774A.1 cells. Chen et al. used HeLa cells to detect MLKL oligomerization and confirmed this result in L929 cells. MLKL translocation to the plasma membrane was measured in MLKL deficient L929 cells using a fluorescent MLKL construct and live cell imaging. It was proposed

that plasmalemmal MLKL complexes then induced either Ca^{2+} (Cai et al., 2014) or Na^{+} (Chen et al., 2014) overload of the cell. However, new studies from Wang's and Vandenabeele's groups have demonstrated that MLKL can bind to phosphatidylinositides (PIPs) and can directly permeabilize liposomes containing these phospholipids (Dondelinger et al., 2014; Wang et al., 2014). Dondelinger et al. initially characterized MLKL's interaction with PIPs using a recombinant MLKL fragment and a lipid array and confirmed its pore forming abilities using liposomes loaded with MLKL. Finally, to confirm MLKL-PIP interactions in necroptosis, L929 and Jurkat cells were treated with PIP production inhibitors, which attenuated TNFα induced necrosis. It is important to note that the early cell free results of MLKL-PIP interactions were not confirmed in whole cells, but instead were modulated indirectly by pharmacologic intervention. Wang et al. confirmed MLKL translocation to the plasma membrane upon induction of necroptosis in HT-29 cells. MLKL-lipid interactions were tested using recombinant protein in a cell free system. And MLKL was shown to be able to form pores in liposomes. Again, however, these results were not confirmed in live cells. These data suggest that it is MLKL itself that is responsible for the final rupture of the plasma membrane during necroptosis and would again rule out a role for mitochondria.

CONCLUSIONS

It is clear that our current understanding of programmed necrosis needs to change. These recent studies would indicate that the execution of programmed necrosis proceeds by a novel, yet to be characterized mitochondrially independent mechanism. However, inspection of all of the literature suggests that cell-specific contexts may occur where the mitochondrial and necroptotic pathways bisect to mediate necrosis. Moreover, even if these pathways are truly distinct, it is unlikely that they will function in isolation in disease. The question is then what is the relative contribution of each pathway to a given necrotic state and how do they coordinate at certain levels.

Additionally, the majority of necroptotic studies have been performed *in vitro* in a variety of cell types, and different cell types may execute necroptosis via different mechanisms. Multiple studies have shown that mitochondrial ROS is critical to the execution of necroptosis in L929 cells (He et al., 2009; Vanlangenakker et al., 2011; Ardestani et al., 2013), while HT29 and U937 cells seem to lack this requirement (Degterev et al., 2005; He et al., 2009). Disparate results have been obtained in MEFs with ROS being both implicated in Lin et al. (2004) and dispensable for Tait et al. (2013) necroptosis. It would be of value to repeat the experiments undertaken by Tait et al. in a cell line in which mitochondrial ROS has been implicated in necroptosis, such as L929 cells, to determine if there is a universal mechanism of necroptotic signaling or if the execution of this pathway is cell-type specific. Whether or not necroptosis requires ROS or mitochondria *in vivo* has yet to be fully determined. For that matter, a majority of the studies performed in the study of necrosome signaling have utilized immortalized cell lines, which begs the question, how well will these findings translate into primary culture systems? Additionally, Ca^{2+} overload induces necrotic cell death. Can this occur in a mitochondria free system? By answering these questions, the actual pathway of necroptosis can be determined. It is possible that there may be multiple routes to necroptosis, and that under certain conditions the mitochondria are not required for its execution.

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Role of β-hydroxybutyrate, its polymer poly-β-hydroxybutyrate and inorganic polyphosphate in mammalian health and disease

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We provide a comprehensive review of the role of β-hydroxybutyrate (β-OHB), its linear polymer poly-β-hydroxybutyrate (PHB), and inorganic polyphosphate (polyP) in mammalian health and disease. β-OHB is a metabolic intermediate that constitutes 70% of ketone bodies produced during ketosis. Although ketosis has been generally considered as an unfavorable pathological state (e.g., diabetic ketoacidosis in type-1 diabetes mellitus), it has been suggested that induction of mild hyperketonemia may have certain therapeutic benefits. β-OHB is synthesized in the liver from acetyl-CoA by β-OHB dehydrogenase and can be used as alternative energy source. Elevated levels of PHB are associated with pathological states. In humans, short-chain, complexed PHB (cPHB) is found in a wide variety of tissues and in atherosclerotic plaques. Plasma cPHB concentrations correlate strongly with atherogenic lipid profiles, and PHB tissue levels are elevated in type-1 diabetic animals. However, little is known about mechanisms of PHB action especially in the heart. In contrast to β-OHB, PHB is a water-insoluble, amphiphilic polymer that has high intrinsic viscosity and salt-solvating properties. cPHB can form non-specific ion channels in planar lipid bilayers and liposomes. PHB can form complexes with polyP and Ca^{2+} which increases membrane permeability. The biological roles played by polyP, a ubiquitous phosphate polymer with ATP-like bonds, have been most extensively studied in prokaryotes, however polyP has recently been linked to a variety of functions in mammalian cells, including blood coagulation, regulation of enzyme activity in cancer cells, cell proliferation, apoptosis and mitochondrial ion transport and energy metabolism. Recent evidence suggests that polyP is a potent activator of the mitochondrial permeability transition pore in cardiomyocytes and may represent a hitherto unrecognized key structural and functional component of the mitochondrial membrane system.

Keywords: inorganic polyphosphate, β-hydroxybutyrate, poly-β-hydroxybutyrate, cardiovascular disease, heart failure, mitochondrial permeability transition pore

WHAT IS β-HYDROXYBUTYRATE?

β-Hydroxybutyrate (β-OHB; also known as 3-hydroxybutyric acid) is a metabolic intermediate that constitutes ∼70% of ketone bodies produced in liver mitochondria mainly from the oxidation of fatty acids released from adipose tissue (Persson, 1970). The term "ketone bodies" refers to three molecules: (1) β-OHB, (2) its dehydrogenated counterpart acetoacetate (AcAc), and (3) the decarboxylated AcAc, acetone (**Figure 1**). Acetone, produced in smaller quantities than the other ketone bodies, is exhaled and essentially is unmeasurable in healthy individual (Laffel, 1999; Cahill and Veech, 2003). AcAc and β-OHB are transported by blood to the extrahepatic tissues, where they are oxidized via the tricarboxylic acid (TCA) cycle to provide the energy required by tissues such as skeletal and heart muscle and the renal cortex (**Figure 2**). In the normal adult heart, mitochondrial oxidative phosphorylation provides more than 95% of the ATP generated for its mechanical, electrical and homeostatic needs. Fatty acid oxidation accounts for up to 70% of the ATP produced by the heart, with metabolism of glucose, lactate, amino acids and ketone bodies supplying the rest (reviewed in Lopaschuk et al., 2010; Cotter et al., 2013). However, ketone body contribution to the overall energy metabolism in the heart and other extrahepatic tissues increases significantly after prolonged exercise, during fasting, adherence to low carbohydrate ketogenic diet (LCKD) and in the neonatal period (Veech et al., 2001; Veech, 2004). In these physiological situations, the concentration of circulating blood β-OHB rises from ∼0.1 mM observed in normal fed state to ∼1 mM after few hours of fasting, and

Abbreviations: AcAc, acetoacetate; AF, atrial fibrillation; BDH, D-β-hydroxybutyrate dehydrogenase; β-OHB, β-hydroxybutyrate; cPHB, complexed poly-β-hydroxybutyrate; FFAR3, free fatty acid receptor 3; FOXO3a, Forkhead box O3a; GPCRs, G-protein-coupled receptors; IDL, intermediate density lipoproteins; HDACs, class I histone deacetylases; HF, heart failure; HMG-CoA, β-hydroxy-β-methylglutaryl-CoA; LCKD, low carbohydrate ketogenic diet; LDL, low density lipoproteins; LFI, low flow ischemia; PHB, poly-β-hydroxybutyrate; polyP, inorganic polyphosphate; SCOT, succinyl-CoA:3-oxoacid-CoA transferase; TCA, tricarboxylic acid; VLDL, very low density lipoproteins.

up to 5–7 mM after prolonged starvation (Cahill, 1970; Robinson and Williamson, 1980; Laffel, 1999; Cahill and Veech, 2003). If the release of free fatty acids from adipose tissue exceeds the capacity of tissue to metabolize them, as occurs during insulin deficiency of type I diabetes or less commonly in the insulin-resistant of type II diabetes, severe and potentially fatal diabetic ketoacidosis can occur where blood β-OHB levels can reach up to 25 mM (Lebovitz, 1995; Cahill and Veech, 2003). Mild elevation of blood ketone bodies also occurs during the process of normal aging (Sengupta et al., 2010) and during congestive heart failure (HF) (Kupari et al., 1995; Lommi et al., 1996, 1997) however it remains unclear whether this elevation represents an adaptive mechanism required to maintain cell metabolism or actually contributes to the progression of disease. Induction of mild states of hyperketonemia may have certain therapeutic benefits (Veech et al., 2001; Veech, 2004; Clarke et al., 2012b). Ketone body oxidation is especially critical in the brain which cannot utilize fatty acids for energy (free fatty acids do not cross the blood-brain barrier) when blood glucose levels become compromised. In this case, ketone bodies provide the brain with an alternative source of energy, amounting to nearly 75% of the brain's energy needs during periods of prolonged fasting and starvation (Owen et al., 1967; Cahill, 2006). It has been known for many years that children with multidrug-resistant refractory epilepsy improve dramatically on a strict LCKD (Veech, 2004), and recent data indicate that β-OHB supplementation protects neurons in models of Alzheimer and Parkinson's disease (Kashiwaya et al., 2000; Tieu et al., 2003; Reger et al., 2004). The laboratory of Veech (Kashiwaya et al., 1994; Sato et al., 1995) was first to report that addition of 4 mM β-OHB to the working perfused rat heart induced an increase in work output but a significant decrease in oxygen consumption. The data demonstrated that the increased efficiency was the result of the widening mitochondrial substrate ratio of NADH and NAD^+ between complex 1 and complex 2 of the mitochondrial respiratory chain. The net effect is a greater potential for ATP production making β-OHB the most efficient fuel in the heart.

Evolutionary, metabolism of ketone bodies is conserved among eukarya, bacteria, and archaea (Reusch, 1992; Aneja et al., 2002; Cahill, 2006). Most bacteria use poly-β-hydroxybutyrate (PHB, **Figure 1**), a polymerized form of β-OHB, as an energy store (Anderson and Dawes, 1990; Reusch, 1992, 2012). PHB consists up to 90% of dry weight in some protozoans. Even archaea use it for energy storage, which suggests it has been around for well over 2–3 billion years. It is possible that its selection was aided by the periods of low environmental oxygen that occurred during the Archaean, Proterozoic, and Palaeozoic eras (Cahill, 2006). PHB is stored as several large granules in the cytoplasm (Anderson and Dawes, 1990; Reusch, 2012), therefore having very little osmotic effect which is in contrast to two other fundamental archaeal energy stores, inorganic polyphosphate (polyP) (Kornberg, 1995; Kornberg et al., 1999) and various polysaccharides (Dawes and Senior, 1973). It has been believed that both polyP and PHB remained only in the prokaryotes, however the raising number of studies demonstrated the presence of both polyP and PHB in mammalian cells, and their important physiological roles. Since only small amounts of PHB and polyP are detected in mammalian cells, it is thought that they do not serve as an energy store (Kulaev et al., 2004) but rather participate in cell signaling and proliferation, regulation of mitochondrial function, channel activity, blood coagulation and inflammation, and bone tissue development. This review discusses the roles of β-OHB, PHB, and inorganic polyP with specific focus on cardiovascular health and disease.

WHAT IS POLY-β-HYDROXYBUTYRATE (PHB)?

PHB (also known as Poly-(*R*)-3-hydroxybutyrate) is a biopolymer consisting of linear chains of β-OHB (**Figure 1**). Three types of PHB with different numbers of β-OHB units and with different functions have been discovered: (i) high molecular weight storage PHB consists of 10,000 to >1,000,000 β-OHB residues (storage PHB), (ii) low molecular weight PHB with medium–chain length consisting of 100–300 residues (oligo-PHB), and (iii) short-chain conjugated PHB (cPHB) in which low numbers of β-OHB residues (≤30) are covalently linked to proteins (see Reusch, 1992, 2012, 2013 for comprehensive reviews). Storage PHB was first discovered in granular inclusion bodies (termed carbonosomes) within the cytoplasm of *Bacillus megaterium* in 1925, and later in a wide variety of archaea and eubacteria, principally those that inhabit soil and water ecosystems (Nuti et al., 1972; Anderson and Dawes, 1990; Poli et al., 2011). PHB is produced by these prokaryotes when carbon sources are freely available but other nutrients are limited, thus PHB is considered to serve as a carbon and energy store in these organisms. PHB located within carbonosomes are covered by a layer of lipids and proteins, which include enzymes involved in PHB synthesis and degradation (Potter and Steinbuchel, 2005; Rehm, 2006; Jendrossek, 2009), have attained considerable commercial importance as ingredients of biodegradable plastics and high-technology materials in the medical field (Wu et al., 2009). PHB of medium-chain length was first discovered in the cytoplasmic membranes of genetically-competent bacteria *Azotobacter vinelandii, Bacillus subtilis* and *Haemophilus influenzae* by Reusch and Sadoff (1983) and later in *Escherichia coli (E. Coli)* (Reusch and Sadoff, 1983; Reusch et al., 1986). Interestingly, that *H. influenzae* and *E. coli* do not accumulate PHB granules. Medium-chain PHB, like storage PHB, is insoluble in water and soluble in chloroform, and non-covalently associated with other molecules. In 1989, medium-chain length PHB was recovered from membranes, mitochondria and microsomes of eukaryotes (Reusch, 1989), and in 1992 from very low density and low density lipoproteins (VLDL and LDL) of human plasma (Reusch et al., 1992). The identity of medium-chain length PHB in representative prokaryotic and eukaryotic organisms was revealed by ^{1}H-NMR spectroscopy by Reusch (1992) and confirmed by Seebach et al. (1994). Medium-chain length PHB has been found associated with inorganic polyP in non-covalent complexes that are postulated to play a role in trans-bilayer transport of cations (Reusch and Sadoff, 1988; Reusch et al., 1995; Das et al., 1997) and deoxyribonucleic acids (Castuma et al., 1995; Huang and Reusch, 1995; Reusch, 2000). Huang and Reusch (1996) discovered short-chain PHB (≤10 residues) covalently bound to specific proteins in the membranes and the cytoplasm of *E. coli*. It is generally thought to consist of ≤30 residues but this remains unclear due to the lability of the ester bond and the paucity of samples examined to date. Unlike storage

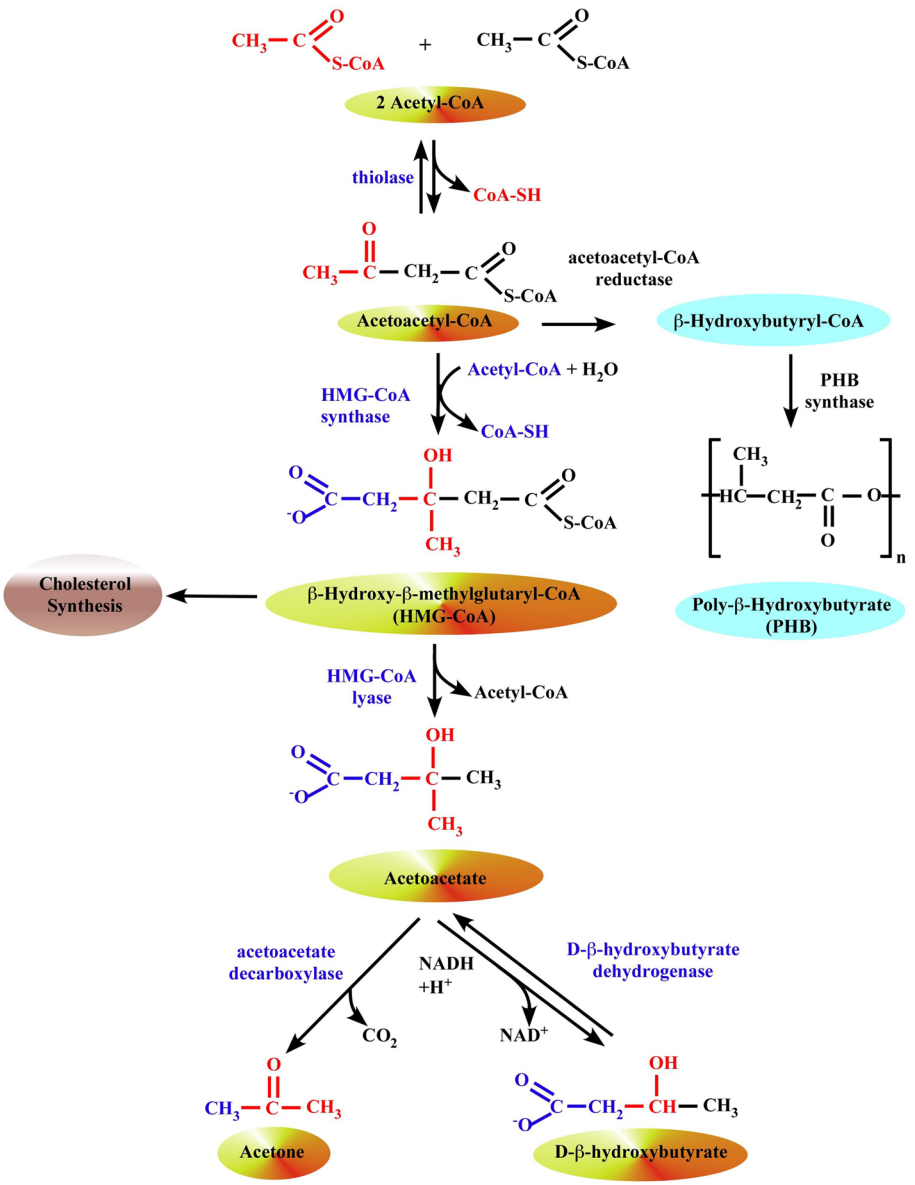

FIGURE 1 | Formation of ketone bodies in liver mitochondria. The synthesis of β-OHB begins with the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA, the parent of the three ketone bodies, by a ketothiolase enzyme. In prokaryotes, this intermediate is subsequently reduced with NADPH to hydroxybutyryl-CoA by acetoacetyl-CoA reductase, and hydroxybutyryl-CoA may then be polymerized to form PHB by the enzyme PHB synthase. In eukaryotes, 3-hydroxy-3-methylglutaryl-CoA synthase (HMG synthase) catalyzes the condensation of acetoacetyl-CoA with a third acetyl-CoA to form β-hydroxy-β-methylglutaryl-CoA (HMG-CoA). The enzyme HMG-CoA lyase then catalyzes the decomposition of HMG-CoA to form acetoacetate and acetyl-CoA, and acetoacetate is further reduced with NADH by phosphatidylcholine-dependent mitochondrial D-β-hydroxybutyrate dehydrogenase to form β-OHB (Lehninger et al., 1960; Marks et al., 1992). Acetoacetate is also non-enzymatically decarboxylated to acetone.

PHB that is segregated within cytoplasmic granules and medium-chain PHB that is "dissolved" in lipid environments, cPHB has been found in all cell compartments of prokaryotes and eukaryotes and in intracellular fluids (Reusch et al., 1997, 2002, 2003; Reusch, 1999; Norris et al., 2009). Indeed, the majority of PHB in cells that do not accumulate long-chain storage PHB is cPHB. It has been postulated that cPHB forms supra-molecular complexes with proteins via covalent bonds and multiple hydrophobic interaction sites (Reusch, 1989, 1999; Seebach et al., 1994). The physical properties of cPHB and large size of the polyester may have substantial structural and functional impacts on the protein. The high-energy C-terminal CoA-ester group, derived from PHB metabolic precursors, presumably acts as a cofactor for the enzymatic reaction in which a covalent bond to the protein is formed (Zhang et al., 2011). This covalent attachment of cPHB is known as PHBylation (Cao et al., 2013) and plays roles in protein folding, protein sorting, or retention of inorganic polyP (Xian et al., 2007; Negoda et al., 2010; Cao et al., 2013). In eukaryotes, cPHB

was found to be bound to the Ca^{2+}-ATPase pump of human erythrocyte membranes (Reusch et al., 1997), which is the sole transporter of Ca^{2+} in red blood cells. Interestingly, inorganic polyP was also present in this complex, and it was suggested that Ca^{2+}-ATPase is a supra-molecular structure consisting of protein, cPHB, and polyP. Solvation of polyP by PHB could allow this polyanion to penetrate into the bilayer portion of Ca^{2+}-ATPase, and possibly mediate Ca^{2+} transfer (Reusch et al., 1997). Recently, it has been found that a member of the transient receptor potential channel family of the melastatin subgroup, TRPM8, which is a major sensor for cold temperatures in the peripheral nervous system, is significantly modified by both cPHB and polyP (Zakharian et al., 2009, 2010; Cao et al., 2013). Moreover, it has been shown that cPHB/polyP complexes isolated from rat liver mitochondria can form voltage-dependent ion channels with multiple conductance states when incorporated in planar lipid bilayers (Pavlov et al., 2005b). The characteristics of this channel activity closely resembled the behavior of the mitochondrial permeability transition pore (mPTP) channel observed in patch-clamp experiments in native mitoplasts (see below for more information). The ubiquitous occurrence of the medium-chain and short-chain polyester (Reusch and Sadoff, 1988; Reusch, 1989, 1992; Reusch et al., 1992, 2003; Seebach et al., 1994; Norris et al., 2009; Zakharian et al., 2009, 2010; Elustondo et al., 2012), suggests that PHB, like polyisoprenoids, polypeptides, polysaccharides, and polynucleotides, is a fundamental constituent of biological cells.

MECHANISMS OF β-OHB/PHB PRODUCTION AND UTILIZATION

Ketone body metabolism includes both ketogenesis and ketolysis (see McGarry and Foster, 1980; Robinson and Williamson, 1980; Cotter et al., 2013 for comprehensive reviews). Ketogenesis is the process by which fatty acids are transformed into AcAc and β-OHB (**Figures 1**, **2**). This process takes place in the mitochondria of liver cells and can occur in response to unavailability of blood glucose (Robinson and Williamson, 1980). The production of ketone bodies is then initiated to make available energy primarily from fatty acids. Fatty acids are enzymatically broken down in β-oxidation to form acetyl-CoA. Under normal conditions, acetyl-CoA is further oxidized and its energy transferred as electrons to NADH and $FADH_2$ in the TCA cycle, and

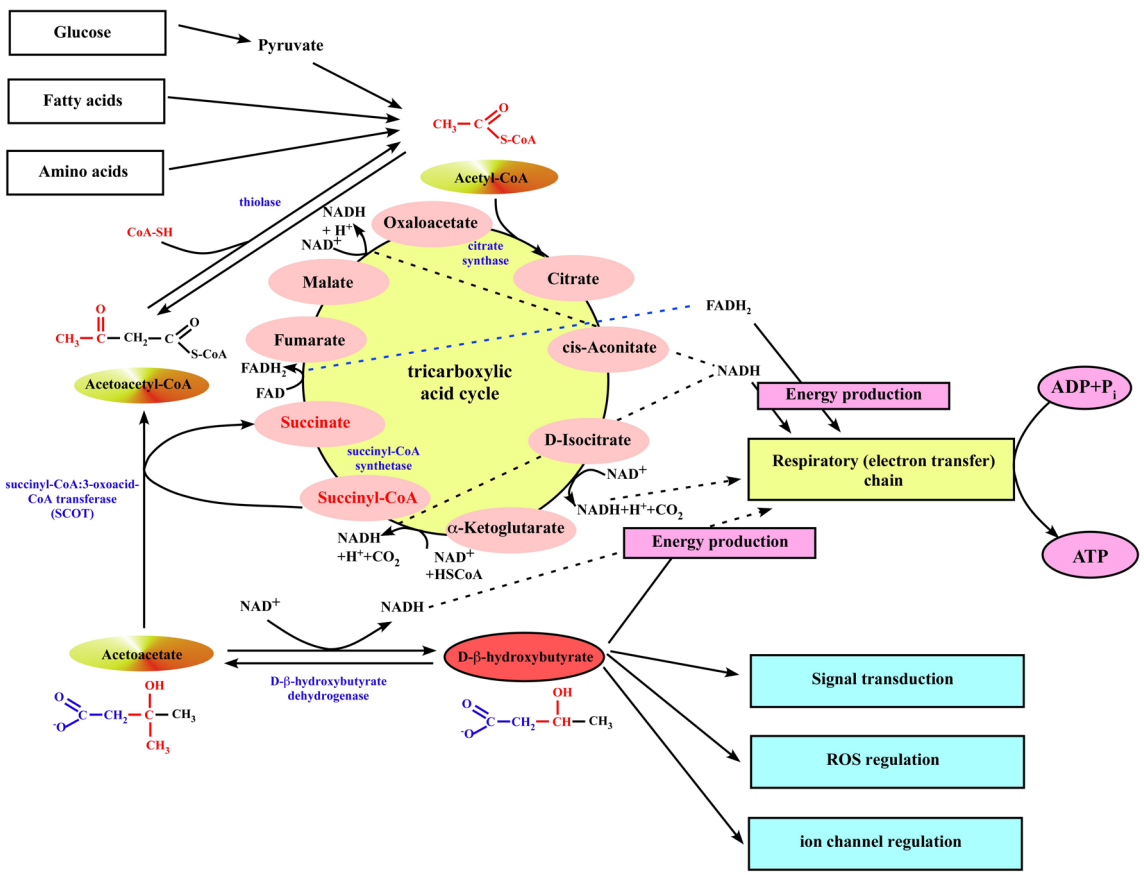

FIGURE 2 | Ketone body utilization in mitochondria of extrahepatic organs. When ketone bodies are delivered to the peripheral organs, β-OHB is oxidized back to acetoacetate by the mitochondrial BDH1. Then, acetoacetate gets converted to acetoacetyl-CoA by the mitochondrial enzyme succinyl-CoA: 3-oxoacid-CoA transferase (SCOT). The succinyl-CoA:3-oxoacid-CoA transferase uses succinyl-CoA as the CoA donor, forming succinate and acetoacetyl-CoA. This reaction bypasses the succinyl-CoA synthetase step of the TCA cycle, although it does not alter the amount of carbon in the cycle. Next, mitochondrial thiolase cleaves the acetoacetyl-CoA into two molecules of acetyl-CoA, which can generate energy by entering the TCA cycle pathway.

further to ATP in the mitochondrial respiratory chain (**Figure 2**). However, if the amounts of acetyl-CoA generated in fatty acid β-oxidation challenge the processing capacity of the TCA cycle or if activity in the TCA cycle is low due to low amounts of intermediates such as oxaloacetate, acetyl-CoA is then used instead in biosynthesis of ketone bodies via acetoacyl-CoA and β-hydroxy-β-methylglutaryl-CoA (HMG-CoA). Deaminated amino acids that are ketogenic, such as leucine, also feed the TCA cycle, forming AcAc and acetyl-CoA, and thereby generate up to 4% of circulating ketones (Merritt et al., 2011). Glucose metabolism accounts for ∼1% of circulating ketones in states of low-carbohydrate intake because pyruvate predominantly enters the hepatic TCA cycle via carboxylation to oxaloacetate or malate rather than decarboxylation (to acetyl-CoA) (Magnusson et al., 1991; Merritt et al., 2011; Jeoung et al., 2012).

The synthesis of β-OHB begins with (i) the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA by a ketothiolase enzyme; this is simply the reversal of the last step of β–oxidation (**Figure 1**). In prokaryotes, this intermediate is subsequently reduced with NADPH to hydroxybutyryl-CoA by acetoacetyl-CoA reductase, and hydroxybutyryl-CoA may then be polymerized to form PHB by the enzyme PHB synthase (Anderson and Dawes, 1990; Poli et al., 2011; Reusch, 2013). (ii) In eukaryotes, 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMG synthase 2) catalyzes the condensation of acetoacetyl-CoA with a third acetyl-CoA to form HMG-CoA (Bahnson, 2004). HMG synthase 2 is exclusively present in liver mitochondria. HMG synthase 1 is located in cytosol and associated with cholesterol biosynthesis. (iii) The enzyme HMG-CoA lyase then catalyzes the decomposition of HMG-CoA to form AcAc and acetyl-CoA, and AcAc is further reduced with NADH by phosphatidylcholine-dependent mitochondrial D-β-hydroxybutyrate dehydrogenase to form β-OHB (Lehninger et al., 1960; Marks et al., 1992). AcAc is also non-enzymatically decarboxylated to acetone. The ratio of β-OHB to AcAc depends on the NADH/NAD^+ ratio inside mitochondria; if NADH concentration is high, the liver releases a higher proportion of β-OHB. Enzymes that polymerize β-OHB or its CoA ester have not yet been identified in eukaryotes (Reusch, 2013). Ketone bodies are released by the liver via solute carrier 16A (SLC16A) family members 1, 6, and 7 and circulate to extrahepatic tissues where they primarily undergo terminal oxidation (Halestrap, 2012; Halestrap and Wilson, 2012; Hugo et al., 2012).

When ketone bodies are delivered to the peripheral organs, β-OHB is oxidized back to AcAc by the mitochondrial D-β-hydroxybutyrate dehydrogenase (**Figure 2**). The utilization of ketone bodies requires an enzyme not present in the ketone body biosynthetic pathway, succinyl-CoA:3-oxoacid-CoA transferase (also known as SCOT), which converts AcAc to acetoacetyl-CoA. This enzyme is not present in liver, and therefore the liver lacks the ability to utilize ketones. The SCOT uses succinyl-CoA in the TCA cycle as the CoA donor, forming succinate and acetoacetyl-CoA. This reaction bypasses the succinyl-CoA synthetase step of the TCA cycle, although it does not alter the amount of carbon in the cycle. Next, mitochondrial thiolase cleaves the acetoacetyl-CoA into two molecules of acetyl-CoA, which can generate energy by entering the TCA cycle pathway (**Figure 2**). This also implies that TCA cycle must be running to allow ketone body utilization.

FEEDBACK REGULATION OF β-OHB SYNTHESIS VIA β-OHB RECEPTORS

β-OHB is a ligand for at least two G-protein-coupled receptors (GPCRs) that bind short-chain fatty acids. HCAR2 (hydroxycarboxylic acid receptor 2; also known as PUMA-G or Gpr109), a $G_{i/o}$-coupled GPCR, first identified as a nicotinic acid receptor (Tunaru et al., 2003), was recently shown to bind and be activated by β-OHB (Taggart et al., 2005). It has been demonstrated that fatty acid derived β-OHB specifically activates HCAR2 receptors within physiologically relevant β-OHB concentrations (K_i = 0.7 mM) (Taggart et al., 2005), typically observed in serum during short-term fasting. Like nicotinic acid, β-OHB reduces lipolysis in mouse adipocytes (with EC_{50} ∼2 mM) possibly creating a negative feedback mechanism to regulate availability of the fatty acid precursors of ketone body metabolism (Taggart et al., 2005; Offermanns et al., 2011). Indeed, in a study of the serum free fatty acids-lowering effect of β-OHB infused in humans, Senior and Loridan (1968) proposed that during starvation ketone bodies exert "a fine regulatory adjustment" of their own synthesis by inhibiting adipocyte lipolysis. β-OHB also binds to and antagonizes the free fatty acid receptor 3 (FFAR3, also known as GPR41), another $G_{i/o}$ protein-coupled receptor that is present in sympathetic ganglions, thereby suppressing sympathetic activity and, in turn, overall metabolic rate in mice (Kimura et al., 2011; Won et al., 2013). Thus, through its actions on HCAR2 and FFAR3, β-OHB may reduce lipolysis, reduce sympathetic tone, and lower metabolic rate.

ROLE OF β-OHB IN CARDIOVASCULAR HEALTH AND DISEASE

The rate of fatty acid oxidation in the normal healthy heart is a function of the arterial free fatty acid concentration and the activities of the enzymes involved in fatty acid transport and oxidation in the mitochondria, specifically carnitine-palmitoyl transferase I (CPT-I) and the enzymes of the β-oxidation pathway (Lopaschuk et al., 1994, 2010; Kunau et al., 1995). The heart readily oxidizes ketone bodies (β-OHB and AcAc) in a concentration-dependent manner at the expense of fatty acid oxidation (Lammerant et al., 1985; Forsey et al., 1987; Stanley et al., 2003). β-OHB has the ability to inhibit lipolysis (Hron et al., 1978), thereby inhibiting the production of the free fatty acid that is implicated in extending myocardial injury by elevating the expression of cardiac mitochondrial uncoupling proteins and decreasing the expression of glucose transporter 4 (Murray et al., 2004; Opie, 2004). In fact, the reduction of concentrations of free fatty acid concentration and the use of alternative substrates was proposed for treatment of patients with HF (Murray et al., 2004). This treatment would reduce mitochondrial uncoupling and restore glucose uptake, thereby improving cardiac efficiency without a fall in cardiac work. In addition, inhibition of fatty acid utilization reduces oxygen demand of adjacent normal myocardial tissue (Lammerant et al., 1985), preventing the extent of cellular damage (Liedtke et al., 1982). Thus, elevated concentrations of β-OHB may prevent myocardial damage by preventing the

formation of damaging intermediates as well as by serving as an alternate energy source (**Figure 2**). Indeed, cardioprotective effects have been observed using *in vivo* ischemia/reperfusion approaches in rats subjected to starvation-induced ketosis, initiated through prolonged fasting, and also via intravenous injection of β-OHB immediately prior to ischemic injury, which conferred a significant decrease in both infarct size and myocardial cell death (Zou et al., 2002; Snorek et al., 2012). Moreover, it has been demonstrated that LCKD enhances cardiac tolerance to global ischemia (Al-Zaid et al., 2007). This study revealed a significant decrease in the number of mitochondria in rats fed a high carbohydrate diet and an increase in the number of mitochondria in those fed a LCKD compared to normal diet group. Rats on LCKD had a remarkable tolerance to ischemia and a faster recovery of cardiac function following reperfusion (Al-Zaid et al., 2007). Transcriptional upregulation of key mediators of mitochondrial oxidative phosphorylation by LCKD significantly extended the lifespan of mice with *Med30*^{zg} mutation (Krebs et al., 2011). Typically, the *Med30*^{zg} mutation causes a progressive and selective decline in the transcription of genes necessary for oxidative phosphorylation and mitochondrial integrity, eventually leading to cardiac failure. A ketogenic diet favorably affected serum biomarkers for cardiovascular disease in normal-weight men (Al-Zaid et al., 2007) and in obese diabetic subjects (Dashti et al., 2007). Cardioprotective effects of β-OHB could be also related to their ability to suppress oxidative stress via transcriptional (Krebs et al., 2011; Shimazu et al., 2013) regulation of key mediators of oxidative stress. The recent study from Eric Verdin's group (Shimazu et al., 2013) demonstrated that β-OHB is an endogenous and specific inhibitor of class I histone deacetylases (HDACs). Inhibition of HDAC by β-OHB was correlated with global changes in transcription, including that of the genes encoding oxidative stress resistance factors Forkhead box O3a (FOXO3a) and MT2. Consistent with increased FOXO3a and MT2 activity, treatment of mice with β-OHB led to substantial protection against oxidative stress. The anti-oxidant effect of β-OHB could be also related to the ability of ketone bodies to oxidize co-enzyme Q (Sato et al., 1995; Veech et al., 2001; Veech, 2004). The major source of mitochondrial free radicals is the half-reduced semiquinone of co-enzyme Q (Chance et al., 1979). Q semiquinine reacts directly with O_2 to form the superoxide radical O^{2-}. By decreasing the reduced form of co-enzyme Q, the mitochondrial production of free radical can be decreased. In a second action of ketone body metabolism, in addition to reducing the mitochondrial NAD^+/NADH redox couple, there is also a reduction of the cytoplasmic free $NADP^+$/NADPH couple. This favors the reduction of glutathione, which is near equilibrium through the action of glutathione reductase (Krebs and Veech, 1969). This is turn would favor the destruction of H_2O_2 by the glutathione peroxidase reaction (Veech et al., 2001; Squires et al., 2003). However, glutathione levels were unchanged during glutamate-induced ROS generation in neurons while ketones inhibited ROS generation by increasing NADH oxidation (Haynes et al., 2003).

While there are many studies which demonstrate that a ketogenic diet results in the improvement of cardiovascular health and significant weight loss, completely opposite results were also reported. For example in an animal study by Wang et al. (2008), rats were fed with LCKD or control diet for 2 weeks and isolated hearts were subjected to normal perfusion in Langendorff mode, with 30 min global low flow ischemia (LFI) followed by 60 min reperfusion, or 60 min LFI followed by 120 min reperfusion. They found that LCKD diet resulted in impaired left ventricular performance during global LFI, reduced recovery of function following LFI and reperfusion, and 10- to 20-fold increased injury as measured by lactate dehydrogenase release and histologic infarct area. Serum FFA, glucose and lactate levels were not different between diet groups, but LCKD did lead to a 2-fold increase in β-OHB (from 0.3 to 0.6 mM) and a 50% decrease in the fed-state insulin level (from 34 to 15 μU/ml) compared to control.

In addition, ketogenic diets can cause biochemical disturbances and cardiac dysfunction in certain vulnerable patients, possibly due to latent defects in ketone body metabolism (Best et al., 2000). In this case study, 20 patients on a ketogenic diet as a treatment for partial seizures and refractory childhood epilepsy were investigated. Prolonged QT interval [with corrected QT (QTc) longer than 450 ms] and cardiac chamber enlargement were found in three pediatric patients (15%). There was a significant correlation between prolonged QTc and both low serum bicarbonate and high β-OHB levels suggesting that the levels of acidosis or ketosis may be important factors in these cardiovascular complications. However, it is important to emphasize that in 17 patients who actually benefited from ketogenic diet (these patients were weaned off anticonvulsants), the serum levels of β-OHB ranged between 4.4 and 6.9 mmol/L (Best et al., 2000). In one patient presented with cardiovascular complications, the serum level of β-OHB of 11.7 mmol/L was detected on admission to the hospital. This excessive elevation of β-OHB definitely can play a role in the development of cardiovascular complications. Cardiovascular disease is a well-known complication of diabetes where levels of β-OHB could reach 25 mM or more in poorly controlled type 1 diabetes (Cahill and Veech, 2003). Age may also play a role, as younger children can have higher β-OHB levels. Some epilepsy patients may have abnormal transcellular membrane ion channels resulting in a predisposition to develop conduction abnormalities as seen in those who develop long QT on cisapride, tricyclics, or class I and III antiarrhythmics (Best et al., 2000). One study revealed that β-OHB at high concentrations (10 mM) reduced L-type Ca^{2+} current in guinea pig ventricular myocytes but only in the presence of the β–adrenergic agonist isoproterenol, however it had no effect on L-type Ca^{2+} current under basal conditions (Kurihara et al., 2012). Another study suggested that the L-stereoisomer of β-OHB could block the transient outward K^+ current (I_{to}) in murine ventricular myocytes causing action potential prolongation (Doepner et al., 1997) while D-β-OHB had no effect. However, this study was later retracted and no other studies are currently available demonstrating possible effects of β-OHB on channel activity in cardiomyocytes. Moreover, hepatic ketogenesis only produces D-β-OHB, which is the only form that is a D-β-hydroxybutyrate dehydrogenase substrate, and thus, a substrate for oxidation (Scofield et al., 1982). L-β-OHB is possibly formed during hydrolysis of the β-oxidation intermediate L-β-OHB-CoA in cardiac mitochondria and does not circulate in the blood (Scofield et al., 1982; Tsai et al., 2006). Interestingly, only the heart contains significant amounts of L-stereoisomer of

β-OHB (Tsai et al., 2006) but its physiological role is unclear. It has been shown that only D-β-OHB was able to inhibit glucose utilization in cardiomyocytes while L-stereoisomer had no effect (Tsai et al., 2006). In a concentration of 5 mM of D-β-OHB, a maximum inhibition effect of 61% of control was found, but L-β-OHB did not interfere with glucose utilization in concentrations from 0.5 to 5 mM. Additionally, when cells were in medium containing L-β-OHB and 5 mM of D-β-OHB, the reduced glucose utilization caused by D-β-OHB gradually recovered with increasing concentrations of L-β-OHB (Tsai et al., 2006).

Currently, very limited data is available on the role of ketone body metabolism in HF. While a mild elevation of blood ketone bodies occurs during congestive HF (Kupari et al., 1995; Lommi et al., 1996, 1997), no changes in cardiac ketone body utilization were detected in humans with advanced HF (Janardhan et al., 2011). It remains unclear whether this elevation represents an adaptive mechanism required to maintain cell metabolism in conditions of HF or actually contributes to the progression of disease. It has been suggested that ketone bodies might be an important fuel during persistent human atrial fibrillation (AF) (Mayr et al., 2008). Proteomic and metabolic analysis of atrial tissue harvested during cardiac surgeries from patients with AF revealed a 2-fold increase in SCOT expression, a mitochondrial matrix enzyme required to activate ketones by transferring CoA from succinyl-CoA to AcAc, the key reaction in ketolytic energy production (see **Figure 2**). Consistent with the proteomic findings, metabolomic analysis revealed a rise in the levels of β-OHB and ketogenic amino acids, notably tyrosine, which forms AcAc and fumarate during catabolism. Fumarate levels were markedly elevated in persistent AF, and consequently the fumarate/succinate ratio increased. The latter is often used as an indicator for the redox state of the coenzyme Q couple (Sato et al., 1995), which is the cofactor for the succinate dehydrogenase reaction and links complex I and complex III of the respiratory chain via its redox span. Both NADH dehydrogenase (complex I) and ubiquinol cytochrome C reductase (complex III) were among the differentially expressed proteins in the proteomic screen. Moreover, animal studies have shown that administration of glucose plus ketone bodies resulted in an increase in the fumarate/succinate ratio similar to the observation in humans with AF (Sato et al., 1995). Interestingly, that elevated myocardial energy expenditure in patients with HF was associated with significant changes in serum metabolomics profiles, especially the concentration of β-OHB, acetone and succinate (Du et al., 2014). Elevated myocardial energy expenditure correlates with reduced left ventricular ejection fraction in HF, and has also been documented as an independent predictor of cardiovascular mortality (Palmieri et al., 2008). Again, it was not investigated whether this elevation in ketone bodies and succinate was a result of adaptation during cardiac remodeling in conditions of HF or represents a maladaptive mechanism contributing to the development of the disease. Definitely, more studies are required to shed light on the role of ketone bodies in cardiovascular diseases. Moreover, to avoid the negative side effects of the ketogenic diets, absorbable ketone body esters comprised of the mono ester of D-β-OHB and R-1,3-butanediol were developed by Veech and colleagues (Clarke et al., 2012a,b). It has been demonstrated that feeding ketone body esters to rats lowers blood glucose and insulin, demonstrating that ketosis actually increases insulin sensitivity (Veech, 2013, 2014). It was suggested that the negative effects of LCKD were associated not with ketone bodies themselves but rather with enhanced release of free fatty acids from the adipose tissue and the correspondent increase in peroxisome proliferators-activated receptor (PPAR) transcription factors with many undesirable effects like development of the atherosclerosis as one example (Kersten et al., 2000).

ROLES OF cPHB IN HEALTH AND DISEASE

Elevated levels of plasma β-OHB observed in HF and diabetes could potentially lead to increased accumulation of cPHB. The pioneering work of Dr. Rosetta Reusch from Michigan State University demonstrated that cPHB could bind to a wide range of proteins modifying the function of voltage-gated ion channels and calcium ATPase pumps (see above and Reusch, 1989, 1992). Dr. Reusch has further demonstrated the importance of cPHB to medicine by showing that cPHB is present in a wide variety of human tissues and also in atherosclerotic plaques (Reusch, 1989, 1992; Reusch et al., 1992; Seebach et al., 1994). However, during digestion processes cPHB may become detached from proteins, and its physical properties—water-insoluble, high intrinsic viscosity, ability to make bilayers non-selectively permeant to ions, sticky (forms multiple non-covalent bonds)—suggest that it may be a factor in the development of some human and animal diseases (Reusch, 1992). Reusch et al. (2003) compared cPHB levels in plasma and tissues of streptozotocin diabetic rats with those in healthy Sprague–Dawley rats. They found 3-to-8-fold increases in cPHB levels in diabetic rats compared to the control animals in the plasma and tissues affected by complications of diabetes—kidney, eye, sciatic nerve, and aorta. These data strongly indicate that cPHB may be an important factor in the development of diabetes, and that plasma cPHB levels may serve as a marker for the disease. Moreover, Norris et al. (2009) proposed that the high intrinsic viscosity of cPHB may play a role in raising intraocular pressure leading to glaucoma. They suggested that cPHB, adhering to filaments in the extracellular matrix, traps debris and triggers aggregation of fibers. This reduces the size of the pores and the flow of the aqueous humor through the meshwork, and thereby raises intraocular pressure.

Using a crotonic acid detection test and cPHB antibodies, Rosetta Reusch was first to demonstrate that cPHB is present in human plasma mainly together with the low-density lipoproteins and with the carrier protein albumin (Reusch et al., 1992). Reusch et al. (1992) determined cPHB levels in plasma of a random group of 24 normal adults and found that concentrations varied from 0.6 to 18.2 mg/l, with a mean of 3.5 mg/l. They further determined that cPHB is carried in lipoprotein particles and albumin—20 to 30% of cPHB is in VLDLs, intermediate density lipoproteins (IDL), and LDLs with most of the remaining 70–80% in albumin. Importantly, they found that cPHB concentrations in plasma correlated strongly with atherogenic lipid profiles. Moreover, no cPHB was associated with HDL. cPHB from ingested food may enter the circulation in the chylomicrons and VLDL similarly to cholesterol (Ramasamy, 2013; Welty, 2013). The amount of cPHB in the VLDL may be a function of

diet, postprandial phase, and genetic factors. Since cPHB cannot be extracted from VLDL with $CHCl_3$, it is considered to be tightly complexed to proteins. The presence of esterases or depolymerases in VLDL is indicated by the lability of cPHB in these particles (Reusch et al., 1992). As VLDL are converted to IDL and then to LDL in the VLDL-IDL-LDL cascade (Ramasamy, 2013), cPHB may be degraded to β-OHB and/or transferred to albumin which solubilizes cPHB and binds it irreversibly (Reusch et al., 1992). Presumably the cPHB-laden albumin is taken to the liver for disposal. cPHB which eludes these disposal mechanisms remains in the LDL. It appears that cPHB accumulates in the denser subgroup of LDL that is most atherogenic (Welty, 2013). It is also noteworthy that cPHB in LDL is $CHCl_3$-soluble, indicating that it is no longer complexed to protein (Reusch et al., 1992). This cPHB, like cholesterol, is likely carried within the lipid core of the LDL particle, which is composed of triglycerides and phospholipids (Reusch et al., 1992; Reusch, 2012). It is this free polyester that may be harmful. As LDL travel through the circulation, some cPHB may be deposited in the arteries and act as a nucleus for the accumulation of cholesterol and other lipids, proteins, salts, etc., thus enhancing atheroma formation (Reusch, 2012). cPHB may also insert into cell membranes, making them non-specifically permeable to ions. In fact the presence of small amounts cPHB was demonstrated in mitochondria isolated from healthy bovine hearts (Seebach et al., 1994). Studies from Pavlov's group (Elustondo et al., 2013a,b; Smithen et al., 2013) addressed the potential role of cPHB in regulation of mitochondrial function. First, they revealed that endogenous cPHB plays a role in mitochondrial Ca^{2+} uptake (Smithen et al., 2013). Mitochondrial PHB depletion achieved by targeted expression of bacterial PHB hydrolyzing enzyme (PhaZ7) significantly inhibited mitochondrial Ca^{2+} uptake stimulated by ATP in intact HepG2, U87 and HeLa cells or by elevated extramitochondrial Ca^{2+}in permeabilized cells. Furthermore, they (Elustondo et al., 2013a,b) demonstrated that addition of synthetic fluorophore-labeled cPHB (fluo-PHB) led to PHB accumulation specifically in mitochondria of cultured HeLa cells. Accumulation of fluo-PHB induced mitochondrial membrane potential depolarization that was delayed by cyclosporin A, de-sensitizer of the mPTP. Moreover, it was demonstrated that fluo-PHB addition caused the transient increase in cytosolic Ca^{2+} concentration in human-derived wild-type SH-SY5Y cells without any affect on mitochondrial Ca^{2+} concentration. However, addition of fluo-PHB to the PINK1 knock-out SH-SY5Y cells which have an impaired mitochondrial Ca^{2+} efflux due to the reduced activity of the mitochondrial Ca^{2+}/Na^{+} exchanger (Gandhi et al., 2009) led to a significant and more sustainable accumulation of Ca^{2+} inside mitochondria. These data indicate that similar to results obtained in phospholipid bilayers of artificial vesicles (Seebach and Fritz, 1999), PHB can increase permeability of plasma and mitochondrial membrane to Ca^{2+} especially under certain pathological conditions. The exact mechanisms of the increased permeability for Ca^{2+} are unknown, however it has been proposed that PHB can act as a natural Ca^{2+} ionophore (Elustondo et al., 2013a). Consideration of PHB as a potential natural ionophore is particularly important in the light of the slow kinetics of mitochondrial ion transport discussed in Kane and Pavlov (2013). Using simple calculations, the authors concluded that the rates of ion transport in intact mitochondria are an order of magnitude lower than can be expected from the estimates based on electrophysiological data obtained in swollen mitoplasts. Specifically: (1) based on direct patch-clamp assays, the Ca^{2+} uniporter is expected to generate a current equivalent to ∼50 pA per single mitochondrion (Kirichok et al., 2004), with some recent papers reporting even higher values (Jean-Quartier et al., 2012; Bondarenko et al., 2013). The estimated maximal uniporter current across the membrane of the functional, intact organelle is expected to be only about 0.1 pA. Furthermore, the uniporter Ca^{2+} flux in the mitochondria of intact cells is estimated to be in the order of 0.005 pA. The presence of slow Ca^{2+} uptake system was in fact suggested to maintain nearly normal animal developmental and physiological function in mitochondrial Ca^{2+} uniporter knock-out mice (Pan et al., 2013). Moreover, it should be noted that cPHB incorporation in membranes would also increase bilayer viscosity, which may negatively influence the performance of membrane proteins (Reusch, 2012, 2013). The fact that mitochondria with highly negative membrane potential across the inner mitochondrial membrane ($\Delta\Psi$ =∼-180 mV) can accumulate fluo-PHB, which is a negatively charged polymer, suggests the existence of an active transport system for PHB. Cyclosporin A sensitivity of PHB-induced mitochondrial membrane depolarization suggests the possible involvement of PHB in activation or regulation of mPTP.

WHAT IS INORGANIC POLYPHOSPHATE?

polyP is a linear polymer of orthophosphate (P_i) residues linked together by high-energy phosphoanhydride bonds as in ATP (**Figure 3A**) (Kornberg et al., 1999). PolyP is present in all living organisms ranging from bacteria to human, and possibly even predating life of this planet (Brown and Kornberg, 2004). The length of polyP chain can vary from just a few phosphates to several thousands phosphate units long, depending on the organism and the tissue in which it is synthesized (Kornberg et al., 1999; Brown and Kornberg, 2004, 2008). PolyP was extensively studied in prokaryotes and unicellular eukaryotes by Kulaev's group in Russian Academy of Sciences (Kulaev et al., 1999a,b) and by the Nobel Prize Laureate Arthur Kornberg at Stanford University (Kornberg et al., 1999; Brown and Kornberg, 2008). In prokaryotes, polyP is synthesized primarily by polyP kinase 1 (PPK1) via transferring the terminal phosphate from ATP to the end of the growing polyP chain (**Figure 3A**), and this reaction is fully reversible and may allow the bacteria to synthesize ATP from stored polyP in times of starvation and environmental stress (Kornberg et al., 1999; Brown and Kornberg, 2004, 2008). Null mutants of PPK1, with low polyP levels, are deficient in survival: namely, they show deficient responses to physical-chemical stresses and predation (Brown and Kornberg, 2004, 2008). Importantly, PPK1 is not the sole polyP-generating enzyme in bacteria since null mutants of PPK1 which lack the ability to produce long-chain polyP (Crooke et al., 1994) still contained a membrane-bound short-chain polyP with 60–70 P_i residues (Castuma et al., 1995). Kornberg's group was the first to identify the presence of a new type of PPK (termed DdPPK2, **Figure 3A**) in *Dictyostelium discoideum* slime mold

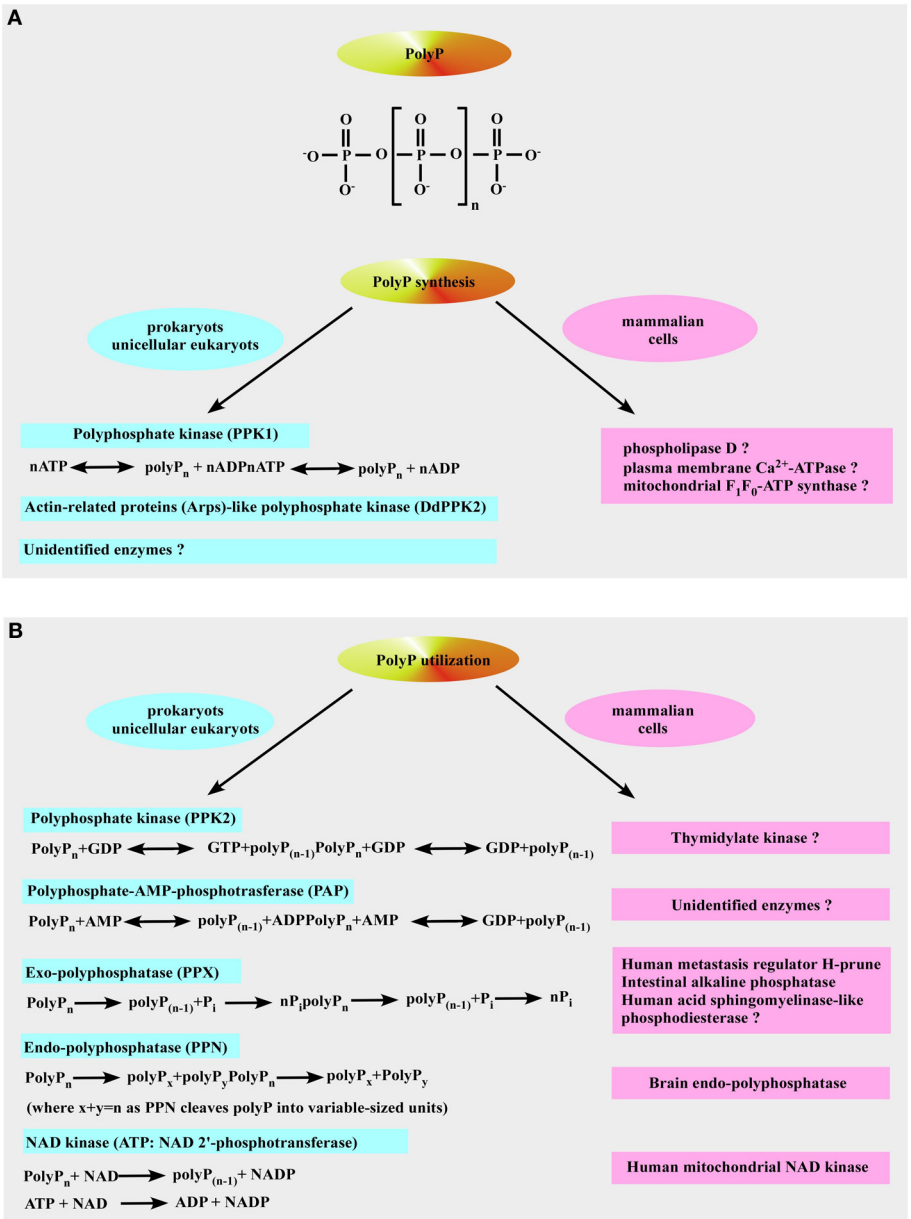

FIGURE 3 | Inorganic polyphosphate structure, formation, and utilization. (A) The upper panel shows the structure of inorganic polyphosphate. The n represents the number of phosphate residues in the polyphosphate chain. It could vary from ten to hundreds of units. The bottom panel is a schematic representation of polyP formation by polyP-generating enzymes in prokaryotes (left) and the putative polyP-generating enzymes in mammalian cells (right). **(B)** Identified pathways and enzymes for polyP utilization in prokaryotes are shown on the left. Mammalian enzymes which resemble the function of polyP-utilizing enzymes in prokaryotes are shown on the right (see text for more details).

(Gomez-Garcia and Kornberg, 2004). Analysis of the single 43-kDa band yielded the remarkable result that the enzyme, DdPPK2, is likely a complex of three actin-related proteins: Arp1, Arp2, and an unreported Arpx that are similar to muscle actins in size and properties. The enzymatic activity of DdPPK2 is highly unusual: (i) synthesis of polyP by DdPPK2 is blocked by actin inhibitors; (ii) this particular Arp complex is an enzyme that can polymerize into an actin-like filament concurrent with its synthesis of a polyP chain in a fully reversible reaction (Spudich, 2004). However, most bacteria synthesize polyP by unknown mechanisms since recent genome screenings through the genome search engine BLAST revealed that PPK1 and PPK2 are present together in less than half of bacterial taxa and that a third of taxa have neither enzyme (Whitehead et al., 2014).

So far, no PPK1 homolog has been identified in higher-order eukaryotes even though it is structurally similar to phospholipase D (Zhu et al., 2005) and, therefore, PPK1 exhibits potential as a novel target for chemotherapy that would affect

both virulence and susceptibility to antibacterial compounds (Brown and Kornberg, 2008). Moreover, it has been demonstrated that plasma membrane Ca^{2+}-ATPase from human erythrocytes may function as a polyphosphate kinase, i.e., it exhibits ATP-polyphosphate transferase and polyphosphate-ADP transferase activities (Reusch et al., 1997). The mitochondrial F_1F_0-ATP synthase can also contribute to polyP generation (see below and Pavlov et al., 2010; Seidlmayer et al., 2012a for details). Two bacterial enzymes [the second PPK (PPK2) and PolyP–AMP–phosphotransferases (PAP)] use polyP as a substrate. PPK2 actually resembles mammalian thymidylate kinase (Whitehead et al., 2013). PAP uses polyP as a substrate to phosphorylate AMP to ADP, an immediate precursor of ATP. PolyP is degraded by both endopolyphosphatases (PPNs) and exopolyphosphatases (PPXs) (**Figure 3B**). In mammalians, a long-chain endopolyphosphatase was purified from rat and bovine brain (Kumble and Kornberg, 1996), a human metastasis regulator protein H-prune was identified as a short-chain specific exopolyphosphatase (Tammenkoski et al., 2008), and mammalian intestinal alkaline phosphatase was characterized as a very active exopolyphosphatase (Lorenz and Schroder, 2001). In addition, 41% homology has been found between yeast exopolyphosphatase PP1 gene product and human acid sphingomyelinase-like phosphodiesterase (Duan, 2006; Kulakovskaya and Kulaev, 2013). In the colon, this enzyme may play anti-proliferative and anti-inflammatory roles via ceramide generation, reducing the lysophosphatidic acid formation, and inactivating the platelet-activating factor and mutations in its gene have been found in cancer cells of the intestines (Duan, 2006). Interestingly, the human protein H-prune exhibits 91% homology with the sequences of yeast exopolyphosphatase PPX1 (Kulakovskaya and Kulaev, 2013). Furthermore, in some prokaryotes [such as *Micrococcus luteus, Corynebacterium ammoniagenes* (Fillipovich et al., 2000), *Micrococcus flavus and Mycobacterium tuberculosis* (Kawai et al., 2000)] NAD kinase catalyzes phosphorylation of NAD using both ATP and polyP as phosphoryl donors (**Figure 3B**) while *E. Coli* NAD kinase is not able to use polyP (Kawai et al., 2001). Remarkably, human mitochondrial NAD kinase has been recently identified to have the ability to utilize both ATP and polyP as the phosphoryl donor (Ohashi et al., 2012). Neglected and long regarded a molecular fossil, polyP has a variety of significant functions in bacteria such as a (i) source of energy (Kulaev, 1979; Wood and Clark, 1988; Kulaev et al., 1999b), (ii) phosphate reservoir (Kulaev et al., 1999b), (iii) donor for sugar and adenylate kinases (Bonting et al., 1991; Hsieh et al., 1993; Phillips et al., 1993), (iv) chelator for divalent cations (Van Veen et al., 1993), (v) buffer against alkaline stress (Pick et al., 1990), (vi) regulator of development (Gezelius et al., 1973), and (vii) structural element in competence for DNA entry and transformation (Reusch and Sadoff, 1988). Even though most of polyP research has been performed in microorganisms, the presence of polyP has been demonstrated in many mammalian tissues (**Figure 4**) such as rodent liver, kidney, lungs, brain, and heart (Kumble and Kornberg, 1995), rabbit heart (Seidlmayer et al., 2012a,b), as well as in human granulocytes (Cowling and Birnboim, 1994), platelets (Smith et al., 2006; Smith and Morrissey, 2008a; Morrissey et al., 2012), and fibroblasts (Pisoni and Lindley, 1992). In striking contrast to microorganisms where polyP is present in millimolar (50–120 mM) concentrations, levels of 25–200 μM (it terms of P_i residues) were found in vast majority of mammalian tissues (Kumble and Kornberg, 1995; Seidlmayer et al., 2012b). The exceptions are platelets and mast cells which contain millimolar concentrations of polyP in electron dense granules (see below). Intracellular distribution of polyP also varies with relatively higher levels of polyP detected in nuclei and plasma membranes isolated from rat liver compared to the cytosol, mitochondria, and microsome fractions (Kumble and Kornberg, 1995). Our studies performed on mitochondria isolated from rabbit hearts detected the presence of ∼200 μM (280 pmol/mg of protein) short-chain polyP with an average chain length of 25 orthophosphates (**Figure 5A** modified from Seidlmayer et al., 2012b). Because polyP is found in small amounts in mammalian cells, it does not serve as phosphate or energy storage (**Figure 4**) but is implicated in cell proliferation (Wang et al., 2003), angiogenesis (Han et al., 2007), apoptosis (Hernandez-Ruiz et al., 2006), osteoblast function (Kawazoe et al., 2004), blood clotting and inflammation (Smith et al., 2006, 2010; Smith and Morrissey, 2008a,b; Muller et al., 2009; Mutch et al., 2010a,b; Choi et al., 2011; Morrissey et al., 2012), cell bioenergetics (Pavlov et al., 2010; Seidlmayer et al., 2012a), ion channel function (Abramov et al., 2007; Kim and Cavanaugh, 2007; Zakharian et al., 2009; Seidlmayer et al., 2012b) and nuclear transcription (Jimenez-Nunez et al., 2012). These new discoveries compelled us to take a fresh look at this natural polymer that has been ignored in biochemistry textbooks for a long time.

PolyP METABOLISM IN MAMMALIAN CELLS AND ROLE OF MITOCHONDRIA

At present very little is known about the molecular details of polyP metabolism in mammalian cells, however it has been demonstrated that newly identified human mitochondrial NAD kinase utilizes not only ATP but also polyP as the phosphoryl donor (Ohashi et al., 2012). It has been postulated for many years that only a subset of bacterial or archaeal NAD kinases exhibit high polyP-dependent NAD kinase activity, while eukaryotic NAD kinases do not (Kawai and Murata, 2008). NAD kinase is the sole $NADP^+$-biosynthetic enzyme known to catalyze phosphorylation of NAD^+ to yield $NADP^+$ and plays a role in the defense against mitochondrial oxidative stress (Pollak et al., 2007). To date no mammalian polyP producing enzymes have been identified, however it has been demonstrated that polyP production in mammalian cells depends on the metabolic state of the mitochondria (Pavlov et al., 2010; Seidlmayer et al., 2012a). Experiments performed on isolated rat liver mitochondria, cultured intact cells (astrocytes, HEK 293) and rabbit cardiomyocytes demonstrated that levels of polyP were increased by substrates of the mitochondrial respiratory chain and in turn reduced by mitochondrial inhibitor (rotenone) or respiratory chain uncouplers [carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) or carbonylcyanide-m-chlorophenylhydrazone (CCCP)]. Oligomycin, an inhibitor of mitochondrial F_1F_0-ATP-synthase, blocked the production of poly P. These data suggest that in mammalian mitochondrial polyP production is closely related to the activity of the oligomycin-dependent F_1F_0-ATP synthase. However, whether or

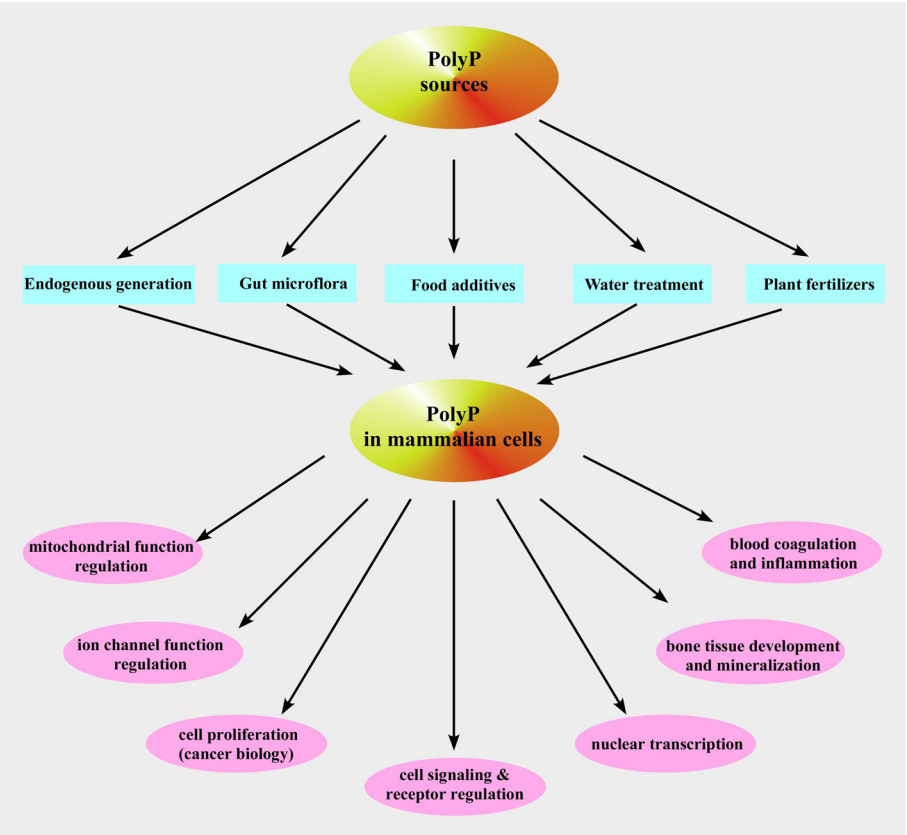

FIGURE 4 | Inorganic polyphosphate sources and functions in mammalian cells. Shown is a schematic representation of the described pathways of polyP occurrence and functions in mammalian cells.

not F_1F_0-ATP synthase is polyP generating enzyme remains to be validated. Furthermore, enzymatic depletion of polyP from cells achieved by overexpression of the mitochondria-targeted yeast polyphosphatase (MTS-GFP-scPPX1) significantly impaired respiratory chain activity (Abramov et al., 2007) and decreased the rate of ATP production which indicates the existence of a feedback mechanism between polyP production and cell energy metabolism (Pavlov et al., 2010). These data are in agreement with earlier studies, where the turnover of polyP demonstrated dependence on the metabolic activity of cells examined (Kumble and Kornberg, 1995). Addition of radioactive $^{32}\mathbf{P}_i$ to cultured cells led to rapid (within minutes to 1 h) phosphate incorporation into polyP in PC12 cells while less metabolically active cells such as embryonic kidney cells and T-cells (Jurkat) showed a significantly slower or no polyP synthesis at all (Kumble and Kornberg, 1995). Furthermore, lysis of cells resulted in a loss of polyP synthetic activity. The authors postulated that polyP synthesis is an energy-dependent process which requires participation of mitochondria (Kumble and Kornberg, 1995; Kornberg et al., 1999).

We investigated the kinetics of mitochondrial polyP metabolism (Seidlmayer et al., 2012a) in intact ventricular cardiomyocytes isolated from control rabbits and animals with HF combined aortic insufficiency and stenosis model (Dedkova et al., 2013). The relative changes in levels of polyP were measured using the fluorescent probe DAPI, with a protocol optimized specifically for polyP detection (Aschar-Sobbi et al., 2008; Seidlmayer et al., 2012b). As demonstrated in **Figure 5B** addition of membrane permeable methyl-succinate—the substrate of the complex II of the mitochondrial respiratory chain—resulted in an increase in DAPI fluorescence by 36 ± 8% ($n = 8$), indicating significant stimulation of the production of mitochondrial polyP (**Figures 5B,C**). On the other hand uncoupling of respiration with FCCP decreased DAPI fluorescence by 29 ± 4% ($n = 8$) presumably due to the stimulation of polyP hydrolysis. This indicates that polyP concentration in cardiac myocytes is variable and depends on levels of energy substrates and the degree of coupling of the mitochondrial respiratory chain. Moreover, we found that polyP metabolism was significantly suppressed in mitochondria of HF myocytes. Addition of methyl-succinate caused only a moderate increase in DAPI fluorescence (16 ± 2%, $n = 10$) (**Figures 5B,C**). Also, the basal polyP levels were significantly lower in conditions of HF (224 ± 21 a.u. in HF vs. 453 ± 80 in control) (**Figure 5D**). This observation raises the intriguing possibility that similarly to bacteria, polyP production in mammalian cells is directly linked to changes in cell metabolism and environment and that diminished polyP synthesis observed in HF myocytes results from the complex remodeling processes during cardiac hypertrophy and HF. A recent study (Gray et al.,

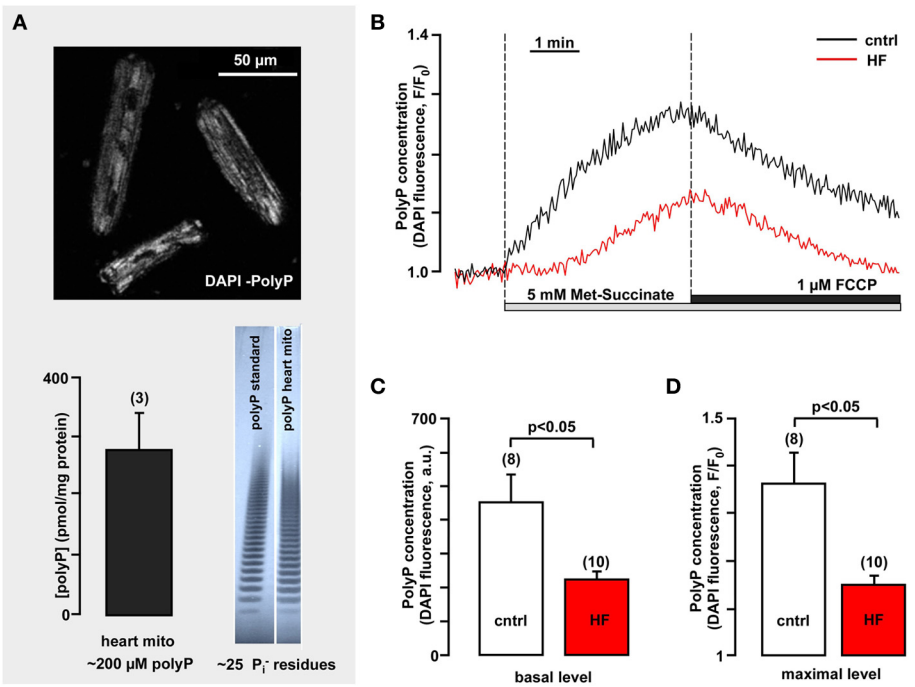

FIGURE 5 | Mitochondrial polyP concentration is highly variable in healthy and heart failure cardiomyocytes and depends on respiratory chain activity. (A) The upper panel image demonstrates polyP detection in freshly isolated rabbit ventricular myocytes using DAPI as a sensor for polyP (λ_{ex} = 408 nm, λ_{em} = 552–617 nm). The bottom panel shows the average amount of polyP in rabbit heart mitochondria (left) and gel images of polyP standard and polyP sample from isolated rabbit mitochondria (right). **(B)** Original recordings of DAPI fluorescence changes in intact cardiac myocytes stimulated with 5 mM methyl-succinate followed by 5 µM FCCP from control (black) and failing myocytes (red). DAPI fluorescence represents changes in polyP concentration. **(C)** Average values of basal DAPI fluorescence in control (black) and heart failure (red) myocytes. **(D)** Average values of maximal DAPI fluorescence after methyl-succinate addition in control (black) and heart failure (red) cells. Modified with permission from Seidlmayer et al. (2012a,b).

2014) determined that in *E. Coli* bacteria polyP acts as an efficient protein chaperon which stabilizes proteins *in vivo*, diminishes the need for other chaperone systems to survive proteotoxic stress (temperature, low pH, oxidants) conditions, and protects a wide variety of proteins against stress-induced unfolding and aggregation. It has been demonstrated that wild type *E. coli* stains generated significant amounts of polyP in response to oxidative stress. This polyP accumulation played a critical role in *E. coli* defense against stress since PPK mutant stains which are not able to produce polyP displayed decreased survival and elevated levels of protein aggregates compared to wild-type strains in response to oxidant or heat stress. PPK mutants showed increased activation of heat shock response, consistent with them suffering from more protein damage upon a similar stress compared to the wild-type. Importantly, we reported a significant increase in reactive oxygen species (ROS) generation and cell death in polyP-depleted ventricular myocytes exposed to simulated ischemia-reperfusion (Seidlmayer et al., 2013) suggesting a protective role of polyP in conditions associated with increased oxidative stress. Furthermore, cell-free experiments revealed that polyP inhibits the aggregation of chemically and heat-denaturated luciferase and citrate synthase, previously established protein chaperon substrates (Gray et al., 2014). PolyP maintained these substrates in a refolding competent state, which required the assistance of the canonical ATP-dependent 70 kilodalton heat shock protein (Hsp70)/DnaK machine. Utilizing standard *in vitro* chaperone assays, it was demonstrated that the presence of 1 µM polyP was sufficient to significantly reduce thermal luciferase aggregation while 100 µM polyP completely prevented aggregate formation. Millimolar concentrations, however, were required to inhibit oxidant or heat-induced aggregation of citrate synthase. Moreover, long-chain polyP polymers (with 130–300 phosphate residues) were more effective as chaperones compared to short chain polymers (~14 phosphate residues). These experiments provide evidence that polyP exerts chaperone activity in concentration- and length-dependent manner and that this activity varies with different substrates. The finding that polyP has stress-protective chaperone activities that resemble the activity of small heat shock proteins is very exciting, however additional research is required to determine the mechanisms of protein aggregation prevention by polyP and the protein targets of polyP in mammalian cells.

INORGANIC PolyP AND ACTIVATION OF THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE (mPTP)

One of the most intriguing and least intuitive roles of polyP is its involvement in membrane ion transport. In 1988 Reusch and Sadoff, using bilayer techniques, demonstrated that genetically competent *E. coli* bacteria contain an ion channel formed by a complex of polyP and PHB (Reusch and Sadoff, 1988).

The channel formed by polyP/Ca^{2+}/PHB interaction was selective for cations with a preference for Ca^{2+}(Reusch and Sadoff, 1988; Reusch et al., 1995). Later a similar polyP/Ca^{2+}/PHB channel was isolated from rat liver mitochondria (Pavlov et al., 2005b). Interestingly, in addition to the cation selective conductance state this mitochondrial complex also demonstrated a high-conductance, weakly-selective, voltage-dependent state. These properties in many ways reflected the behavior of the mPTP as seen in patch-clamp studies of native mitochondrial membranes (Kinnally et al., 1991; Szabo and Zoratti, 1991). Interestingly, the polyP/Ca^{2+}/PHB channel of bacterial origin also has this high conductance state (Pavlov et al., 2005a) and the transition of the channel into a high conductance state would most likely be deleterious for bacterial organisms, raising the question whether most of the time the bacterial channel is either closed or is in the low conductance cationic state. The different bacterial conductance states are reminiscent of conductance states proposed for the mPTP (Ichas et al., 1997; Huser et al., 1998; Ichas and Mazat, 1998). The parallels between bacteria and mitochondria also suggest that similar cationic channels may play a role in normal mitochondrial function. In support of such notion the polyP/Ca^{2+}/PHB complex has been detected in various eukaryotic organisms and cellular compartments suggesting a potential physiological role (Reusch, 1989). Currently, the direct test whether a polyP/Ca^{2+}/PHB complex indeed forms the pore part of the mPTP in intact mitochondria remains an experimental challenge. Nonetheless, the idea that the presence of polyP in intact mitochondria is an essential condition for mPTP opening remains an intriguing hypothesis. Indeed, it was shown that mitochondria of cultured cells with reduced levels of polyP are more resistant toward Ca^{2+}-induced mPTP opening (Abramov et al., 2007). In our work performed in rabbit ventricular cardiomyocytes we demonstrated that polyP depletion with MTS-GFP-scPPX1 overexpression effectively prevented Ca^{2+}-induced mPTP opening (**Figures 6A,B** modified from Seidlmayer et al., 2012b). In contrast to non-excitable cells (Abramov et al., 2007), polyP depletion did not affect the ability of mitochondria to accumulate Ca^{2+}, however significantly increased the resistance of cardiac mitochondria to open mPTP (**Figure 6C**) and prevented Ca^{2+}-induced loss of mitochondrial membrane potential (**Figure 6D**) indicating that polyP is a potent activator of Ca^{2+}-induced mPTP. On the other hand, when mPTP activity was monitored in conditions of simulated ischemia-reperfusion accompanied by massive ROS generation, polyP depletion was not able to prevent mPTP opening and cell death during reperfusion. In fact, as we demonstrated earlier (Seidlmayer et al., 2013), ROS generation and cell death was significantly increased under conditions of ischemia-reperfusion in polyP-depleted cells. We found different modes in mPTP activity during ischemia and reperfusion, and that these modes were affected differently by polyP. In agreement with our data obtained on permeabilized cells (Seidlmayer et al., 2012b), polyP depletion prevented Ca^{2+}-induced low conductance mPTP mode observed during ischemia, however it did not affect ROS-induced mPTP opening in the high-conductance mode during reperfusion. These exciting findings indicate that polyP has a dual effect on mPTP activity—promoting the transient opening of Ca^{2+}-induced mPTP opening which can prevent mitochondria from Ca^{2+} overload. On the other hand, polyP was required for protection against oxidative stress-induced mPTP opening and cell death. It is unclear at this point, whether this effect of polyP was related to the recently discovered chaperone activity of polyP or the direct effect of polyP on mPTP. Recent data suggest that dimers of the of F_1F_0-ATP synthase can form channels with characteristics similar to the mPTP (Giorgio et al., 2013), however the molecular details of channel formation by F_1F_0-ATP synthase remain unclear. Particular attention has been brought to the subunit c of the F_1F_0-ATP synthase as a potential component of the mPTP (Azarashvili et al., 2014; Bonora et al., 2013). Interestingly, an interaction of polyP/Ca^{2+}/PHB complex with subunit c of F_1F_0-ATP synthase was reported back in 2005 (Pavlov et al., 2005b), and therefore it is possible that polyP could provide a fine tuning of mPTP regulation or actually mediate Ca^{2+} transfer through mPTP.

PolyP IS A LINK BETWEEN HUMAN GASTROINTESTINAL TRACT BACTERIA, β -OHB AND CARDIOVASCULAR HEALTH

It has recently been discovered that human gastrointestinal tract bacteria (probiotics) produce polyP, and that polyP is responsible for probiotic actions that protect the intestinal epithelia from oxidant stress and improve epithelial injury due to excess inflammation (Segawa et al., 2011). It has been shown that polyP specifically binds to integrin β1, and inhibition of integrins or p38 MAPK pathway eliminates the protective effect of polyP on intestinal epithelia (Segawa et al., 2011). P38 MAPK is a class of mitogen-activated protein kinases responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock and osmotic shock, and is involved in cell differentiation and apoptosis. Indeed, protective effect of polyP was associated with its ability to induce expression of the cytoprotective heat shock protein 27 (Hsp27) in these cells (Segawa et al., 2011) and to decrease staurosporine-induced apoptosis as evidenced by the inhibition of caspase-9 and caspase-3 activation. Althogether, these results suggest that polyP develops a robust intestinal barrier function through interaction with integrin β1, followed by the p38 pathway activation. Intriguingly, recent studies (Lam et al., 2012; Gan et al., 2014) demonstrated that probiotic administration attenuates myocardial infarction following ischemia-reperfusion injury and myocardial hypertrophy and HF following myocardial infarction in the rat. Lam and coworkers (Lam et al., 2012) provided the first evidence that probiotics may be cardioprotective by showing that administration of a commercially-available beverage containing the probiotic *Lactobacillus plantarum* 299 v 24 h before subjecting rats to 30 min of cardiac ischemia and 2 h reperfusion, produced a 27% reduction in infarct size and improved reperfusion by 35%. In the study of Gan et al. (2014), rats were subjected to 6 weeks of sustained coronary artery occlusion and administered the probiotic *Lactobacillus rhamnosus* GR-1 or placebo. They found that animals administered GR-1 exhibited a significant attenuation of left ventricular hypertrophy and improved hemodynamic parameters. Serial echocardiography revealed significantly improved left ventricular parameters throughout the 6 week follow-up period including a marked preservation of left ventricular ejection fraction as well as fractional shortening.

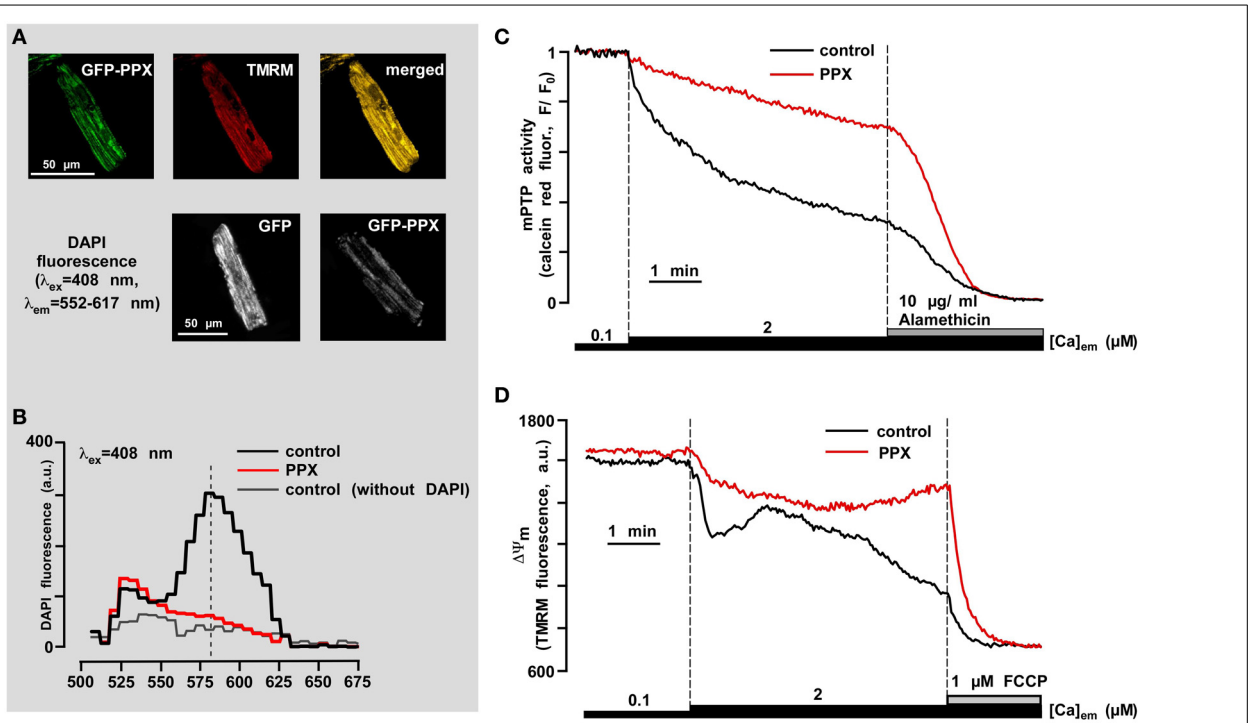

FIGURE 6 | PolyP depletion prevents opening of the permeability transition pore induced by mitochondrial Ca^{2+} overload. (A) The upper panel shows co-localization of GFP-PPX signal with mitochondria. TMRM was used as a mitochondrial signal and the degree of overlay is presented in shades of yellow in the merged image. The bottom panel shows the decrease in DAPI fluorescence in polyP-depleted cells. **(B)** Fluorescence spectrum of DAPI (5 μM) loaded myocytes expressing control GFP (black), PPX (red), and control GFP cells not loaded with DAPI (gray). **(C)** Original recordings of mPTP opening using calcein red release from mitochondria of permeabilized control (black) and polyP-depleted (PPX expressing, red) myocytes. After permeabilization cells were exposed to 2 μM Ca^{2+} and 10 μg/ml alamethicin was added at the end of the experiment to achieve the maximal calcein red release from mitochondria. **(D)** Original recordings of mitochondrial membrane potential ($\Delta\Psi_m$) with the voltage-sensitive dye TMRM in permeabilized cells upon elevation of the $[Ca^{2+}]_{em}$ from 0.1 to 2 μM, and subsequent addition of 1 μM FCCP in control (black) and polyP-depleted (PPX expressing, red) myocytes. Modified with permission from Seidlmayer et al. (2012b).

Beneficial effects of GR-1 were still evident in those animals in which GR-1 was withdrawn at 4 weeks suggesting persistence of the GR-1 effects following cessation of therapy. Investigation of mechanisms showed a significant increase in the leptin to adiponectin plasma concentration ratio in rats subjected to coronary ligation which was abrogated by GR-1. Metabonomic analysis showed differences between sham control and coronary artery ligated hearts particularly with respect to preservation of myocardial taurine levels. These studies suggest that gut microbiota can modulate cardiovascular disease possibly due to alterations in the production of gut-derived hormones which exert cardiovascular effects. Specifically, both studies detected a significant decrease in leptin production in probiotic-treated animals. PolyP levels were not measured in these studies, however, it is plausible to speculate that polyP produced by gut microbiota could also exerts a cardioprotective role for the host organism. Interestingly, germ-free mice which were born and raised in sterile gnotobiotic isolators, had a significantly reduced heart weight (Crawford et al., 2009) compared to those of normally colonized mice. Metabolic and physiological studies revealed that functional parameters (heart rate, hydraulic work, mitochondrial morphology, number, and respiration, plus ketone body, fatty acid, and glucose oxidation) of the hearts of germ-free mice were relatively normal in fed conditions. However, a significant decrease in hepatic ketogenesis and levels of circulating β-OHB was detected under fasting conditions in germ-free mice with a compensatory increase in cardiac glucose oxidation (Crawford et al., 2009). The reduction in heart size and alterations of myocardial metabolism were reversed in germ-free mice maintained on a ketogenic diet or following microbial colonization (Crawford et al., 2009). Altogether, the data demonstrate the existence of a link between gut microbiota and cardiovascular health, however the exact roles of polyP and β-OHB in cardiovascular health and disease remain to be determined. It is intriguing to speculate that polyP produced by gut microbiota can modulate signaling cascades and ketone body metabolism. Importantly, mammalian intestinal alkaline phosphatase has been described as a very active exopolyphosphatase (Lorenz and Schroder, 2001), which can split long-chain polyP produced by gut microbiota into short-chain polyP and phosphate. The short-chain polyP could be efficiently absorbed in human intestinal tract and excreted in the urine (Karp et al., 2012). A recent study (Karp et al., 2012) compared the effect of polyP and phosphate supplementation in healthy women 19–31 years of age and revealed that both monophosphate and polyP induced an increase in serum parathyroid hormone and serum/urine phosphate levels, while only polyP led to a significant

decrease in urinary Ca^{2+} levels. This decrease in Ca^{2+} levels most likely was due to enhanced Ca^{2+} binding to polyP in intestine and decreased Ca^{2+} absorption (Zemel and Linkswiler, 1981). In mammals, circulating inorganic phosphate and polyP (complexes with Ca^{2+}) serve to support extracellular mineralization, which appears to furthermore depend on the concerted expression of collagen type 1 and tissue-non-specific alkaline phosphatase (Murshed et al., 2005). To control mineralization and cellular delivery, extracellular phosphate levels and total body phosphate content are tightly regulated by a number of hormones, including parathyroid hormone, 1,25-dihydroxy-vitamin D, and fibroblast growth factor-23 (*FGF23*), and serum phosphate feeds back to regulate these factors in an endocrine fashion (Civitelli and Ziambaras, 2011): high phosphate increases the secretion of parathyroid hormone and *FGF23*, while low phosphate stimulates the synthesis of 1,25-dihydroxy-vitamin D. Hypophosphatemia leads to demineralization of the skeleton (osteomalacia), whereas hyperphosphatemia is an important risk factor for the development of vascular calcifications (Rutsch et al., 2011). Interestingly, it has been shown recently that polyP actually stimulates *FGF23* expression through activation of fibroblast growth factor receptor (Sun et al., 2012), and promotes bone mineralization (Hacchou et al., 2007). Moreover, while polyP of different chain length (short, medium, and long) stimulated *FGF23* expression, polyP with long chains actually led to the increased lactic acid accumulation and cell death (Sun et al., 2012). Furthermore, high levels of circulating *FGF23* are linked to the increased incidents of pathological cardiovascular events (Arnlov et al., 2013).

ROLES OF PolyP AND β-OHB IN CELL PROLIFERATION—IMPLICATIONS FOR CANCER BIOLOGY

It has been demonstrated that addition of polyP to human plasma cells produced an unexpected inhibition of immunoglobulin secretion and stimulation of apoptosis. PolyP induced apoptosis specifically in human plasma cells, myeloma (malignant plasma cells) cell lines, primary myeloma cells, and B lymphoid cell lines (Hernandez-Ruiz et al., 2006). Normal B cells, T cells, total blood mononuclear cells, and non-lymphoid cell lines were not affected by polyP. In the U266 myeloma cell line, polyP induced externalization of phosphatidylserine, activation of caspase-3, and arrest of the cell cycle. The protective effects of interleukin-6 did not overcome the polyP-induced apoptosis. This study, however, used very high (3–6 mM) concentrations of polyP with various chain lengths (25, 45, and 75 P_i residues), and detected that in these concentrations polyP effectively inhibited immunoglobulin secretion, initiated apoptosis and reduced cell survival of the U266 myeloma cell line. Since total peripheral blood mononuclear cells, T cells, B cells, and non-lymphoid cell lines were not affected, it was suggested that polyP could potentially be useful in the design of new antimyeloma drugs. Also, polyP could contribute in part to maintaining low levels of plasma cells in blood circulation (Jimenez-Nunez et al., 2012).

Another study (Han et al., 2007) demonstrated that polyP effectively blocked *in vivo* pulmonary metastasis of B16BL6 cells by suppression of neovascularization, whereas it did not affect proliferation or adhesion to extracellular matrix proteins. PolyP not only inhibited bFGF (basic fibroblast growth factor)-induced proliferation and ERK (extracellular-signal-regulated kinase)/p38 MAPK (mitogen-activated protein kinase) activation of human endothelial cells, but also blocked the binding of bFGF to its cognate cell surface receptor. Furthermore, polyP inhibited bFGF-induced *in vitro* and *in vivo* angiogenesis, suggesting that polyP possesses an anti-angiogenic activity. Since neovascularization is essential for tumor metastasis, these findings indicate that polyP has an *in vivo* anti-metastatic activity via its anti-angiogenic activity.

Interestingly, ketogenic diet lowered the serum ratio of IGF/IGF-binding protein 3 in mice with positive effects on metabolic syndrome and cancer risk (Freedland et al., 2008). Also, ketogenic diet (and elevated β–OHB) increased AMPK in mice, inhibiting the mTOR/Akt signaling pathway (McDaniel et al., 2011). However, polyP was also found to stimulate mTOR signaling and possibly cancer growth (Wang et al., 2003). In this study, polyP depletion achieved by overexpression of yeast exopolyphosphatase *PPX1* gene inhibited proliferation of the human breast carcinoma cell line MCF-7 via mTOR signaling cascade. These conflicting results definitely should stimulate further research to determine the exact roles of polyP and β–OHB in cell growth and cancer biology. Recently, human metastasis regulator protein H-prune was identified as a short-chain specific polyP hydrolase (Tammenkoski et al., 2008). Long-chain polyP (>25 phosphate residues) were converted more slowly, whereas pyrophosphate and nucleoside triphosphates were not hydrolyzed. Notably, the exopolyphosphatase activity of H-prune was suppressed by long-chain polyphosphates and pyrophosphate, which could be potential physiological regulators. Clearly, the chain length of polymer determines the physiological role of polyP making it either a friend or foe.

ROLE OF PolyP IN BONE TISSUE DEVELOPMENT AND MINERALIZATION

The vertebrate skeleton is predominately composed of bone, a mineralized tissue that consists of type-1 collagen, non-collagenous proteins and a calcium phosphate mineral known as apatite (Omelon et al., 2009). Unlike invertebrate skeletons or protective shells, the vertebrate skeleton is continually rebuilt, repaired and resorbed during growth, normal remodeling, and recovery from traumas and diseases. Even though the role of polyP in the modulation of bone mineralization was suggested nearly 15 years ago (Leyhausen et al., 1998), only recently considerable advances were made in the study of polyP contribution to bone tissue development (Kawazoe et al., 2004; Hacchou et al., 2007; Omelon et al., 2009; Morimoto et al., 2010; Usui et al., 2010). It has been shown that polyP is present abundantly in normal human osteobalsts, at bone-resorbing osteoclastic sites, in the proliferating and hypertrophic zone chondrocytes, in the hypertrophic zone matrix, and in unmineralized osteoid (Shiba et al., 2003; Kawazoe et al., 2008). Moreover, polyP enhanced alkaline phosphatase activity and expression of osteopontin and osteocalcin genes in MC3T3-E1 osteoblastic cells suggesting that polyP promotes calcification in these cells (Kawazoe et al., 2004). Interestingly, polyP was detected in electron-dense Ca^{2+}-rich granules localized at sites of bone resorption (Omelon et al., 2009). The size of these dense granules was larger (in the range

of a few thousand angstroms) than previously reported for mitochondrial electron-dense clusters in mineralizing osteoid (ranging from 400 to 1000 angstroms) (Landis et al., 1977) The authors (Omelon et al., 2009) put together a hypothesis that apatite mineral dissolution in the osteoclast resorption zone increases the concentrations of free P_i and Ca^{2+}. The mitochondria may scavenge the P_i and condense it into polyP that may also sequester Ca^{2+}. Amorphous granules containing total concentrations of Ca^{2+} and P_i higher than the saturation of apatite are formed and may be transported out of the osteoclast. The granules may be transported to or produced within the osteoblasts that build new bone. The osteoblasts may embed the granules in osteoid (new, unmineralized bone). The reaction of these granules with alkaline phosphatase (present at the membrane of osteoblasts) cleaves P_i from polyP and would increase the free P_i concentration and release any sequestered Ca^{2+}. The increase in free Ca^{2+} and P_i could exceed the saturation for apatite and result in apatite mineral formation. Most recently (Tsutsumi et al., 2014), it was demonstrated that polyP promotes MC3T3-E1 cell maturation from a resting state to an active blastic cell phase which implies that polyP can be an effective material for bone regeneration. The enzyme responsible for polyP synthesis in bone tissue cells has not been identified and the signal molecules determining separate stages of this process are unknown. Fascinating, *in vivo* experiments demonstrated that artificial introduction of polyP into the bone growth or regeneration zone accelerates these processes (Grynpas et al., 2002; Pilliar et al., 2007; Shanjani et al., 2007; Doi et al., 2014). The finding that polyP induces osteoblastic differentiation and bone mineralization creates a basis for development of novel polyP-containing drugs for bone disease treatment and new polyP-containing materials for bone substitution.

ROLE OF PolyP IN BLOOD COAGULATION AND INFLAMMATION

It has been known for many years that polyP accumulates in many infectious microorganisms (Kornberg et al., 1999) and therefore could play a significant role in inflammation. Microbial polyP (with chain length of more than 500 P_i residues), which is highly pro-coagulant, may function in host responses to pathogens (Chuang et al., 2013). Of particular interest to hematology, it has been demonstrated that polyP is a major component of dense granules of human platelets (Ruiz et al., 2004) and acidocalcisomes of mast cells (Moreno-Sanchez et al., 2012), where millimolar levels (in terms of P_i residues) of short chain polyP (~70–75 phosphate units in platelets and 60 phosphate units in mast cells) were detected. Like acidocalcisomes, human platelets dense granules are spherical, acidic, electron-dense, and contain large amounts of calcium and potassium in addition to polyP (Ruiz et al., 2004). Both human platelets and mast cells secrete polyP upon activation which indicates that polyP could be an important mediator of their pro-inflammatory and pro-coaguant activities. Indeed, studies from Morrissey's lab and others have shown that polyP is a potent modulator of the blood clotting cascade, acting as a pro-hemostatic, pro-thrombotic and pro-inflammatory agent depending on its polymer size and location (Smith et al., 2010). PolyP may represent at least one of the long-sought patho-physiologic activators of the contact pathway of blood clotting, and its actions may also help to explain previously unexplained abilities of activated platelets to enhance plasma clotting reactions. Targeting polyP with phosphatases interfered with pro-coagulant activity of activated platelets and blocked platelet-induced thrombosis in mice (Muller et al., 2009). Addition of polyP restored defective plasma clotting of Hermansky–Pudlak Syndrome patients, who lack platelet polyP (Muller et al., 2009). Remarkably, both Hermansky–Pudlak (HPS) and Chediak–Higashi (CHS) syndromes have in common dense granule deficiency and bleeding tendency (Salles et al., 2008), which is congruent with the presence of poly P in dense granules (Ruiz et al., 2004) and its role in blood clotting (Smith et al., 2006). The data identify polyP as a new class of mediator having fundamental roles in platelet-driven pro-inflammatory and pro-coagulant disorders. PolyP acts via several points in the clotting cascade: (i) it triggers clotting via the contact pathway (Smith et al., 2006, 2010; Muller et al., 2009), (ii) it accelerates factor V activation (Smith et al., 2006), (iii) it enhances fibrin clot structure (Smith and Morrissey, 2008b; Mutch et al., 2010a), and (iv) it accelerates factor XI back-activation by thrombin (Mutch et al., 2010b; Choi et al., 2011). It was demonstrated that very long polymers (with more than 500 P_i residues, such as those present in microorganisms) were required for optimal activation of the contact pathway, while shorter polymers (with ~100 P_i residues, similar to the polymer lengths released by platelets) were sufficient to accelerate factor V activation and abrogate the anticoagulant function of the tissue factor pathway inhibitor. Optimal enhancement of fibrin clot turbidity by polyP required polyP with more than 250 P_i residues. Therefore, polyphosphate of the size secreted by platelets is very efficient at accelerating blood clotting reactions but is less efficient at initiating them or at modulating clot structure (Smith et al., 2010). PolyP may prove useful as a hemostatic agent to control bleeding, and conversely, polyP antagonists might be beneficial as anti-thrombotic/anti-inflammatory agents with reduced bleeding side effects. Several polyphosphate inhibitors have been identified recently and their effectiveness was tested *in vitro* and*in vivo* (Smith et al., 2012). Polyphosphate inhibitors were anti-thrombotic in mouse models of venous and arterial thrombosis and blocked the inflammatory effect of polyphosphate injected intra-dermally in mice. This provides proof of principle for polyphosphate inhibitors as anti-thrombotic/anti-inflammatory agents *in vitro* and *in vivo*, with a novel mode of action compared with conventional anticoagulants (Smith et al., 2012). However, the detailed molecular mechanisms by which polyP modulates blood clotting reactions still remain to be elucidated (Morrissey, 2012).

PolyP REGULATES INNATE IMMUNITY BY MODULATING iNOS EXPRESSION IN MACROPHAGES– SIGNALING ROLES OF PolyP

It has been proposed recently that endogenous polyP may serve as an intercellular signaling molecule in innate immunity (Harada et al., 2013). As we discussed above, both platelets and mast cells store polyP in their granules and secrete it into the extracellular space when the cells are activated (Ruiz et al., 2004; Moreno-Sanchez et al., 2012). Moreover, it has been shown that parasites such as *Trypanosoma cruzi (T. cruzi)* contain polyP

within molar levels in acidocalcisomes which is essential for the parasite to resist the stressful conditions in the host and to maintain a persistent infection (Galizzi et al., 2013). Interestingly, infection with *T. cruzi* leads to the development of chronic Chagas heart disease characterized by autonomic nervous system derangements, microvascular disturbances, parasite-dependent myocardial infection, and immune-mediated myocardial injury (Marin-Neto et al., 2007). Despite nearly one century of research, the pathogenesis of chronic Chagas cardiomyopathy is incompletely understood (Marin-Neto et al., 2007). Tightly packed polyP may be released into the extracellular space and affect the function of macrophages, thereby serving as an immune modulator. Indeed, the recent study (Harada et al., 2013) demonstrated that polyP suppressed inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) release induced by lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, in mouse peritoneal macrophages. In contrast, polyP did not affect the LPS-induced release of TNF, another inflammatory mediator. Using polyP of various chain lengths (14, 60, and 130 P_i residues) it was demonstrated that polyP with longer chains (130 P_i residues) was more potent than those with shorter chains in suppressing LPS-induced iNOS expression. It has been shown that polyP suppressed LPS-induced iNOS expression by down-regulating the level of mRNA expression, however the detailed mechanisms are currently unknown. LPS-induced extracellular Ca^{2+} influx enhances iNOS expression in macrophages (Kim et al., 2004; Zhou et al., 2006). PolyP, a negatively charged polyanion, may bind with extracellular Ca^{2+}, inhibit LPS-induced Ca^{2+} influx, and result in a decreased expression of iNOS mRNA (Harada et al., 2013). On the other hand, extracellular polyP has been recently reported to modulate or activate receptors on the plasma membrane, such as the FGF, integrin, and $P2Y_1$ receptors (Shiba et al., 2003; Segawa et al., 2011; Holmstrom et al., 2013). Potentially, polyP may regulate iNOS expression via modulation of these receptors.

CONCLUSIONS

The main objective of this review was to emphasize the importance of ketone body β-OHB, its natural polymer PHB, and inorganic polyP in cardiovascular physiology and other diseases. While physicians were always taught that elevation in β-OHB levels is undesired, recent studies indicate that a mild elevation in β-OHB levels could be actually beneficial in certain physiological situations, and can modulate the important signaling cascades involved in cell growth, proliferation and defense against oxidative stress. Two ancient polymers, PHB and polyP, have proved to be involved in regulation of protein channels in plasma membrane and mitochondria, and should not be discarded as "relics" from Prebiotic times.

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The "Goldilocks Zone" from a redox perspective—Adaptive vs. deleterious responses to oxidative stress in striated muscle

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Consequences of oxidative stress may be beneficial or detrimental in physiological systems. An organ system's position on the "hormetic curve" is governed by the source and temporality of reactive oxygen species (ROS) production, proximity of ROS to moieties most susceptible to damage, and the capacity of the endogenous cellular ROS scavenging mechanisms. Most importantly, the resilience of the tissue (the capacity to recover from damage) is a decisive factor, and this is reflected in the disparate response to ROS in cardiac and skeletal muscle. In myocytes, a high oxidative capacity invariably results in a significant ROS burden which in homeostasis, is rapidly neutralized by the robust antioxidant network. The up-regulation of key pathways in the antioxidant network is a central component of the hormetic response to ROS. Despite such adaptations, persistent oxidative stress over an extended time-frame (e.g., months to years) inevitably leads to cumulative damages, maladaptation and ultimately the pathogenesis of chronic diseases. Indeed, persistent oxidative stress in heart and skeletal muscle has been repeatedly demonstrated to have causal roles in the etiology of heart disease and insulin resistance, respectively. Deciphering the mechanisms that underlie the divergence between adaptive and maladaptive responses to oxidative stress remains an active area of research for basic scientists and clinicians alike, as this would undoubtedly lead to novel therapeutic approaches. Here, we provide an overview of major types of ROS in striated muscle and the divergent adaptations that occur in response to them. Emphasis is placed on highlighting newly uncovered areas of research on this topic, with particular focus on the mitochondria, and the diverging roles that ROS play in muscle health (e.g., exercise or preconditioning) and disease (e.g., cardiomyopathy, ischemia, metabolic syndrome).

Keywords: hormesis, carbonyl stress, mitochondria, lipid peroxidation, redox environment, adaptation, heart, skeletal muscle

INTRODUCTION

A fairy tale emerged in England during the first half of the nineteenth century, telling the story of how a meddlesome little girl named Goldilocks discovered the forest home of a family of bears. In this classic tale, the reader will remember that during her exploration of the bears' home, Goldilocks determined that baby bear's porridge was "not too hot, not too cold," and his bed was "not too hard, not too soft." In essence, they were "just right." Such is the nature of homeostasis in our organ systems, in that stress in either one direction or another can tip the scales toward disease, or maladaptation, thereby disrupting homeostasis. It is in this context that the redox environment of a cell is maintained within a "Goldilocks Zone," whereby ROS production is sufficiently counterbalanced by the antioxidant capacity/quality control systems, and the environment is optimal for homeostasis. However, when faced with persistent or uncompensated oxidative stress, the redox environment can be pushed outside of this Goldilocks Zone, where cell death, inflammation and disease ensues. The schematic shown in **Figure 1A** illustrates this concept of the Goldilocks Zone in redox balance, and uses two contrasting stressors that are common to the cardiovascular system, cardio-metabolic disease and exercise, as examples. Both exercise and disease generate oxidative stress in striated muscle. However, the persistent and uncompensated oxidative stress resulting from cardio-metabolic disease ultimately pushes the myocytes outside the Goldilocks Zone, while the pulsatile, compensated oxidative stress that follows successive bouts of exercise keeps the myocytes in the Zone, and in good health.

High metabolic demand in striated muscle necessitates a far greater degree of arterial blood perfusion and oxygen tension than virtually any other tissue in the body. The natural consequence of this high availability and concentration of oxygen is

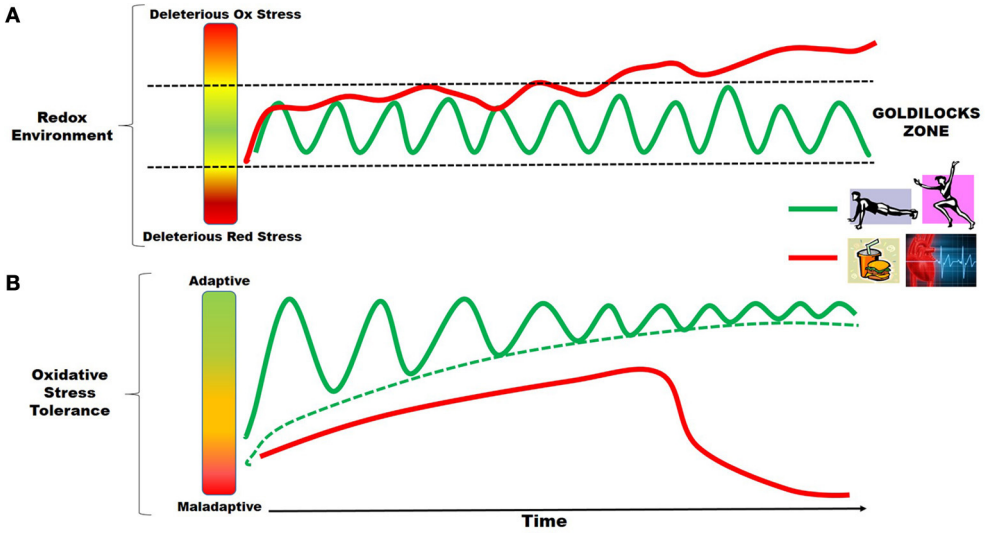

FIGURE 1 | Oxidative stress adaptations in striated muscle and the Goldilocks Zone. The concept of the Goldilocks Zone in the redox environment of striated muscle is illustrated in the schematics shown above. Time-dependent changes in tissue oxidative stress due to exercise (green line) is pulsatile in nature, coming from consecutive bouts of exercise over time, whereas the oxidative stress arising from cardio-metabolic diseases (red line) is persistent. As depicted in **(A)**, the ultimate outcome of these two stressors is divergent, since exercise-induced ROS over time keeps the myocytes well within the Goldilocks Zone of homeostasis (region between the Reducing and Oxidative stress, two dashed black lines), while disease-induced ROS eventually pushes the myocytes outside of the Zone, leading to deleterious consequences (e.g., unresolved inflammation, electro-mechanical dysfunction, mitochondrial dysfunction and cell death). These divergent outcomes are best explained by the adaptive responses in the antioxidant capacity and cell quality control mechanisms that are elicited by these two sources of oxidative stress. **(B)** Illustrates this adaptive response, in that exercise-induced ROS leads to augmentation of the antioxidant capacity and protection against exogenous stressors over time. Conversely, disease-induced ROS, due to its persistent and insidious nature, ultimately overwhelms endogenous protective mechanisms, ultimately resulting in a maladaptive response.

a greater burden placed upon endogenous antioxidant systems to buffer the ROS that are invariably produced as a bi-product. Numerous enzymatic and non-enzymatic sources of ROS exist in skeletal muscle and heart, and it is only through the work of these important antioxidant networks that the cellular redox environment is maintained within a range that is optimal for homeostasis and proper electro-mechanical function. ROS production in striated muscle is persistent in nature, therefore, oxidative stress in this tissue should be viewed in the context of a continuum in that there is always a degree of oxidative stress present, and it only becomes deleterious under certain contexts (i.e., persistence, compartmentalization or lack of adaptive responses). In fact, recent work in transgenic mice has documented that the removal of NADPH oxidase (NOX) in the heart, and the subsequent presence of overwhelming reductive stress, can lead to exacerbation of ischemia/reperfusion injury (Matsushima et al., 2013). A similar phenomenon is observed in skeletal muscle, where overexpression of the antioxidant enzyme glutathione peroxidase-1 (GPx1) in mice was shown to cause insulin resistance (McClung et al., 2004). These paradoxical findings embody the "not too hot, not too cold" concept of the Goldilocks Zone in redox balance, and also underscore both the complexity and importance of the redox environment to homeostasis. Further, they are evidence of the need for a more rigorous investigation of the mechanisms by which the redox environment of striated muscle adapts to various metabolic challenges.

This paradox between "good" and "bad" oxidative stress is best exemplified in the contrasting effects of the increased ROS that is well-known to occur in striated muscle with exercise, compared to the increased ROS from cardio-metabolic diseases characterized by metabolic supply/demand mismatch, such as nutrient overload (i.e., obesity, high fat/high sugar diets, diabetes) and ischemia. Shown in **Figure 1B** is a schematic depicting how disease-induced ROS and exercise-induced ROS lead to divergent outcomes in striated muscle. Both cardio-metabolic disease and exercise cause increases in ROS, but they differ in their substance and temporality. The ROS produced from successive bouts of exercise is pulsatile in nature, with periods of recovery in between (green line **Figure 1B**). Indeed, increased ROS occurs in skeletal muscle and heart during exercise from a variety of sources, but the broad outcome of this oxidative stress is beneficial, resulting in the induction of mitochondrial biogenesis and aerobic capacity, augmented antioxidant capacity, improved vascularization, and insulin sensitivity. The beneficial ROS-mediated adaptations to exercise have been the topic of many thorough reviews (Powers and Jackson, 2008; Powers et al., 2010, 2011a,b; Gomez-Cabrera et al., 2012). In many ways the response of striated muscle to exercise-induced oxidative stress is embodied in the concept of hormesis, in that the muscle experiences a beneficial, adaptive response to sub-lethal increases in ROS that occur during and immediately following each bout of exercise (Ristow and Schmeisser, 2011; Nikolaidis et al., 2012). Conversely, nutrient overload and ischemia/reperfusion also cause increased ROS in skeletal muscle and heart from numerous sources, particularly mitochondria, but net effects are deleterious, resulting in inflammation

and recruitment of immune cells, activation of pro-fibrotic gene expression and cardiac hypertrophy, increased cell death, and insulin resistance (reviewed in Fisher-Wellman and Neufer, 2012; Cavalera et al., 2014; Dai et al., 2014). Some evidence of modest hormesis has been reported in myocardium with nutrient overload/diabetes (Joyeux-Faure et al., 2006; Tocchetti et al., 2012; Fisher-Wellman et al., 2013; Rindler et al., 2013; Chen et al., 2014; Lejay et al., 2014), but ultimately these modest adaptive responses become overwhelmed, and the tissue/organ succumbs to the deleterious effects over time (red line in **Figure 1B**).

The "Goldilocks Zone" of redox balance in muscle scales down from whole tissue to mitochondrial level. Work by Aon and colleagues showed that rates of ROS emission (ROS produced—ROS scavenged) from mitochondria are substantially elevated when the mitochondrial redox environment is either highly reduced (e.g., nutrient overload, diabetes), or highly oxidized (e.g., reperfusion) (Aon et al., 2010; Cortassa et al., 2014). This increased ROS emission observed at both ends of the redox spectrum would presumably lead to different consequences at cell and tissue level, as the antioxidant capacity is considerably more compromised during a highly oxidized state than it is during a highly reduced state. At the level of cells and tissues, however, there are also many other considerations such as the temporality of the change in redox environment (i.e., acute vs. persistent) and the existing mechanisms of adaptation intrinsic to the cells experiencing the stress at a given time, all of which would dictate the global response to increased mitochondrial ROS emission.

In the following sections we provide an overview of this contrast in outcomes between the "bad" oxidative stress that occurs in skeletal muscle and heart as a result of diseases associated with metabolic supply/demand mismatch (e.g., nutrient overload/obesity, ischemia), and "good" oxidative stress that occurs in these organs with therapeutic interventions such as pre-conditioning and exercise. Major sources of ROS and their byproducts in these conditions will be discussed, along with potential mechanisms underlying the adaptive, beneficial responses and the maladaptive, deleterious responses. Emphasis will be placed on mitochondrial sources of ROS and mitochondrial antioxidant networks, as this organelle is recognized as the primary source, sink, and target of intracellular ROS in muscle. Furthermore, descriptions of lesser-known types of oxidative stress (e.g., lipid peroxidation and carbonyl stress) and relevant detoxification systems are another area of special emphasis, as these are increasingly recognized to be major players in ROS-mediated adaptation. Where appropriate, clinical and translational studies will be described.

CARDIO-METABOLIC DISEASE AND OXIDATIVE STRESS IN STRIATED MUSCLE

Much focus has been directed in recent years toward documenting the contribution of ROS "production" to disease etiology, and toward identifying enzymatic sources of ROS as underlying mechanisms. Such an emphasis toward the "supply-side" of ROS is important, but can often be short-sighted as it ignores the vast redox circuits and antioxidant networks that exist within cells. Indeed, a major source of the problem of oxidative stress in chronic disease likely involves the inability of the myocytes' antioxidant networks to adequately compensate for the increase in ROS production that occurs. Never-the-less, for purposes of this portion of the review we will focus on major sources of ROS production within myocytes, since a number of sources have consistently been shown to be associated with cardio-metabolic diseases such as obesity, diabetes, myocardial ischemia, and other chronic diseases. Whether this increased ROS is cause or consequence of these diseases is still not clear, although it appears likely that it is both. **Figure 2** illustrates some of the major sources of ROS in myocytes which will be discussed in the subsequent sections, as well as common deleterious responses and adaptive responses that accompany the ROS. Understanding the mechanisms behind this lack of adaptability, or maladaptive response to ROS, may be the key to unraveling this paradox of "good vs. bad" oxidative stress in striated muscle, thereby leading to improvements in existing therapies and identification of novel therapeutic targets.

MAJOR SOURCES OF ROS PRODUCTION IN STRIATED MUSCLE WITH CARDIO-METABOLIC DISEASES

Prominent mitochondrial and non-mitochondrial enzymatic sources of ROS

The cytosol, plasma membrane, nucleus, and peroxisomes are all sites of ROS production, but for myocytes in particular, the mitochondria is widely considered to be the predominant source of ROS (reviewed in Jezek and Hlavata, 2005). Within the mitochondria, the electron transport system (ETS) has been well-studied as a source of ROS production with superoxide radicals forming spontaneously at complexes I, II, and III via the swift addition of leaking electrons to an incompletely reduced oxygen (St-Pierre et al., 2002; Kudin et al., 2004; Drose and Brandt, 2012; Siebels and Drose, 2013). A comprehensive overview of the sites and regulation of ROS production by the mitochondrial ETS is beyond the scope of this review, and has been extensively reviewed elsewhere (Liu, 1997; Andreyev et al., 2005; Stowe and Camara, 2009).

Although the ETS is unquestionably a major producer of ROS in myocytes, the enzyme monoamine oxidase (MAO) is often overlooked as a mitochondrial source of ROS in cardiomyocytes. Tethered to the outer mitochondrial membrane, MAO is responsible for oxidative deamination of catecholamines, simultaneously generating H_2O_2, NH_4^+ and reactive aldehydes in the process (Lewinsohn et al., 1978). The two isoforms of the enzyme, MAO-A and MAO-B, have differing affinities for various substrates and inhibitors, although both isoforms can metabolize all catecholamines to some extent. MAO-A is selectively inhibited by clorgyline and has been shown to have a significantly greater capacity to metabolize serotonin and norepinephrine than MAO-B. MAO-B is selectively inhibited by selegiline and has a profoundly greater capacity to metabolize phenylethylamine then MAO-A. Both isoforms contribute relatively equally to oxidative deamination of dopamine, tyramine, and tryptamine (Youdim et al., 1988). The predominance of one isoform vs. the other within the body is tissue-dependent and the levels between species can also vary significantly (Saura et al., 1996). The heart

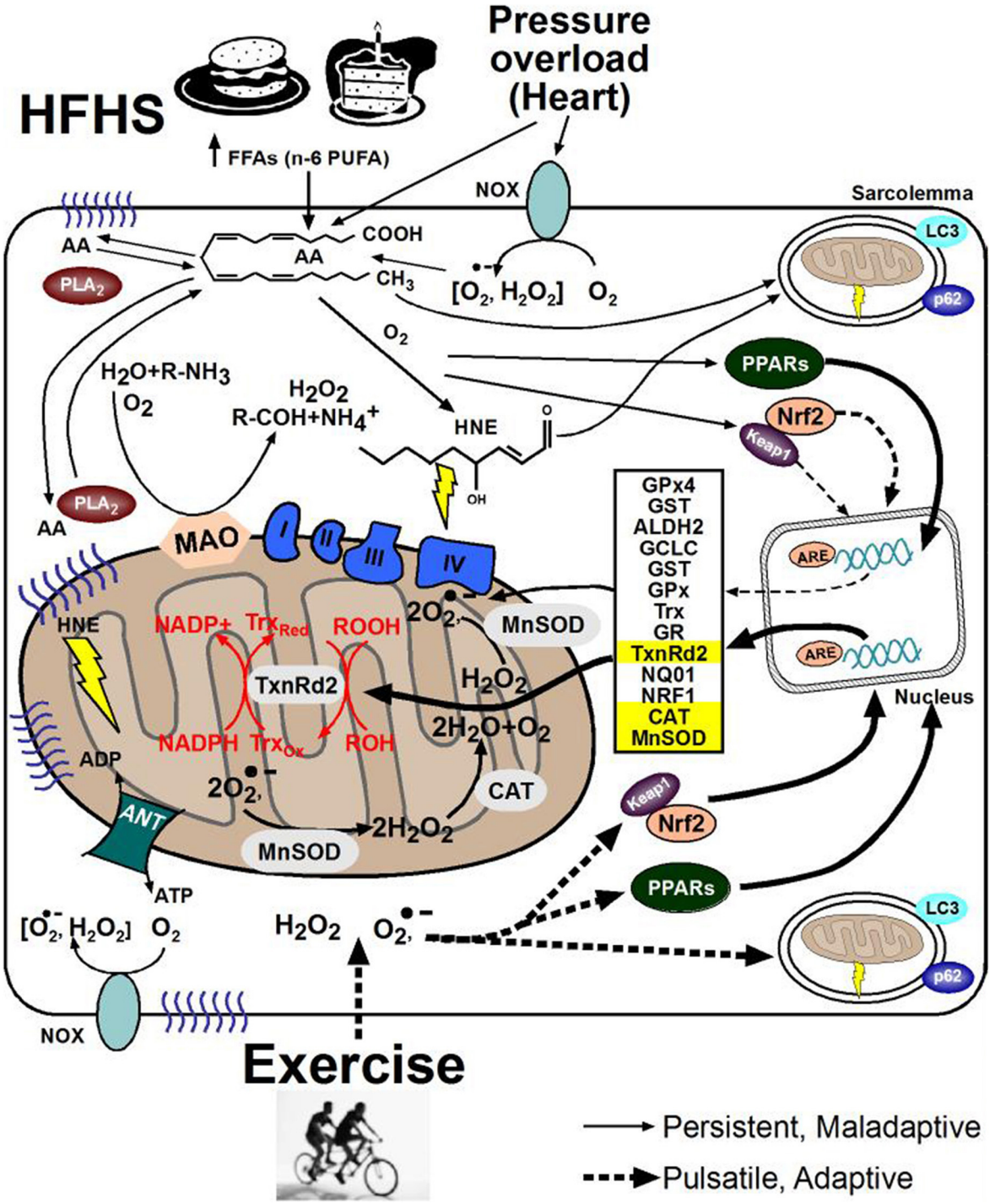

FIGURE 2 | Cell signaling pathways underlying ROS adaptation in striated muscle. The beneficial and deleterious consequences of oxidative stress coming from cardio-metabolic diseases (top) or exercise (bottom) in striated muscle at the subcellular level are shown here. Note the difference in designation of solid-line arrows for the persistent ROS coming from disease, compared to dashed-line arrows representing the pulsatile ROS coming from exercise. Formation of lipid peroxides and their derivative aldehydes such as 4-hydroxynonenal (HNE), along with their subsequent reactivity to cause protein carbonylation and carbonyl stress is depicted by yellow lightning bolts in cytosol and mitochondria. (NOX, NADPH oxidase; HFHS, high fat, high sucrose; PUFA, polyunsaturated fatty acids; FFA, free fatty acids; AA, arachidonic acid; PLA_2, phospholipase A_2; PPAR, peroxisome proliferator-activated receptor; ANT, adenine nucleotide translocase; COX IV, cytochrome oxidase IV; ATP, adenosine tri-phosphate; Nrf2, NF E2-related factor 2; NRF1, nuclear respiratory factor 1; GST, glutathione S-transferase; ALDH2, aldehyde dehydrogenase 2; GCLC, γ-Glutamylcysteine ligase catalytic subunit; GPx, glutathione peroxidase; NQO-1, NADPH-quinone oxido-reductase-1; GR, glutathione reductase; GSHt, total glutathione; Trx, thioredoxin; TxnRd2, thioredoxin reductase-2; MAO, monoamine oxidase; MnSOD, manganese superoxide dismutase; CAT, catalase).

contains a mix of both isozymes corresponding to its metabolic needs, much of which is a result of the continuous exposure to dopamine and norepinephrine from nerve terminals and circulating epinephrine from the adrenal glands (Kaludercic et al., 2011, 2014). A recent study completed in our laboratory showed that MAO activity is highly variable between individuals and that it is a major generator of ROS in human atrial myocardium (Anderson et al., 2014a).

MAO has both direct and indirect ROS-producing capacity. It has been shown that in isolated brain mitochondria, MAO directly generates a 48-fold greater rate of H_2O_2 than the ETS (Hauptmann et al., 1996). The H_2O_2 produced is converted into water and molecular oxygen by peroxidases, and the aldehyde metabolite produced is efficiently converted by aldehyde dehydrogenase (ALDH) into its respective carboxylic acid. In mitochondria, ALDH2 has been shown to be responsible for converting the highly reactive aldehyde metabolites of dopamine oxidation (3,4-dihydroxyphenylacetaldehyde, DOPAL), and serotonin (5-hydroxyindole-3-acetaldehyde, 5-HIAL) into their corresponding acetates (Ambroziak and Pietruszko, 1991; Rooke et al., 2000). When MAO activity is high, a surge in catecholamine levels would be expected to cause greater ROS production from the ETS, suppressed ATP production, insufficient calcium handling, and mitochondrial DNA damage due to formation of these reactive byproducts if there is not an appropriate antioxidant buffering capacity present.

In addition to mitochondrial sources, the enzyme NADPH oxidase (NOX) is a major producer of ROS in striated muscle. NOX enzyme expression has been documented in the heart at various stages of development, with NOX4 isoform predominating in early developmental stage (Li et al., 2006), and NOX2 in the adult heart (Ambroziak and Pietruszko, 1991). Sakellariou and co-workers recently showed that NOX activity constituted the bulk of ROS production in skeletal myofibers at rest and during contraction, corresponding to expression of NOX2 and NOX4 isoforms in this tissue (Sakellariou et al., 2013).

Lipid peroxidation—A unique and important ROS

The mitochondrial membranes contain a high density of polyunsaturated fatty acids (PUFAs) and phospholipids which are susceptible to peroxidation (Bacot et al., 2003; Osman et al., 2011). Phosphatidylethanolamine and phosphatidylcholine are the most abundant phospholipids and comprise ∼40% and ∼30% of total mitochondrial phospholipids, respectively. Cardiolipin and phosphatidylinositol (PI) account for ∼10–15% of phospholipids, whereas phosphatidic acid (PA) and phosphatidylserine (PS) comprise ∼5% of the total mitochondrial phospholipids. Cardiolipin is concentrated in the inner mitochondrial membrane in close proximity to the ETS. Although it comprises a relatively a low percentage of total membrane phospholipid, its peroxidation is directly associated with a decline in activity of respiratory complexes III and IV, and the release of cytochrome c which initiates apoptotic signaling (Fry and Green, 1981; Paradies et al., 1999; Chicco and Sparagna, 2007). This is significant because cardiolipin is especially susceptible to peroxidation.

Upon formation, lipid peroxides either propagate to form more lipid peroxides, or spontaneously degenerate to form more stable lipid peroxide products (LPPs) (Bridges et al., 1983; Esterbauer et al., 1991). The final LPP formed is dependent on the initial fatty acid oxidized. One particularly reactive LPP formed via peroxidation of n-6 PUFAs such as arachidonic acid (AA, illustrated in **Figure 2**) is the aldehyde 4-Hydroxynonenal (HNE). This is particularly relevant because the Western diet contains a high ratio of n-6 to n-3 PUFAs, ∼10:1 by some estimates (Guichardant et al., 2004; Cordain et al., 2005). HNE is relatively stable, electrophilic and lipophilic thus is capable of traversing across cellular compartments. Approximately 2–8% of HNE formed reacts with proteins as well as DNA- guanine residues to form HNE-adducts (Nair et al., 1999; Siems and Grune, 2003; Blair, 2008; LoPachin et al., 2009; Minko et al., 2009; Maier et al., 2010; Chavez et al., 2011).

DELETERIOUS (i.e., MALADAPTIVE) CONSEQUENCES OF PERSISTENT OXIDATIVE STRESS WITH CARDIO-METABOLIC DISEASES

The presence of a persistent sub-lethal oxidative stress in striated muscle has been associated with a variety of cardio-metabolic diseases for many years. Recent studies have uncovered potential roles for oxidative stress as causal factors underlying these diseases, although extracting clearly defined roles for disease causality vs. simple association remains a challenge. Due to its primary function in regulating cellular metabolism and oxygen consumption, the mitochondrial ETS is known to increase ROS production in striated muscle with a wide variety of diseases involving metabolic supply-demand mismatch. In experimental and clinical models of obesity/type 2 diabetes (O/T2D), an increase in ROS production from mitochondrial ETS has been reported in both heart (Boudina and Abel, 2007; Anderson et al., 2009, 2012; Boudina et al., 2009) and skeletal muscle. During ischemia, mitochondrial ROS is increased from several sites, largely a result of oxidative damage to ETS and to enzymes in the matrix such as aconitase. Upon reperfusion following ischemia, a burst of ROS from the ETS is a well-characterized phenomenon that has been implicated to be both beneficial under some contexts (e.g., pre-conditioning, see next section), or deleterious. Comprehensive reviews of current knowledge regarding the sources and consequences of mitochondrial ROS during ischemia and reperfusion in striated muscle can be found elsewhere (Tullio et al., 2013; Chen et al., 2014; Lejay et al., 2014).

MAO's unique orientation relative to the ETC provides the ability for MAO to interfere with mitochondrial function and cellular energetics, although very little is known about the potential contribution of MAO-derived ROS in cardio-metabolic diseases. One study reported that MAO-derived ROS led to diminished state 3 and state 5 respiration (Cohen and Kesler, 1999). Furthermore, Hauptmann and colleagues showed that MAO-derived H_2O_2 can introduce mutations in mitochondrial DNA (mtDNA). Their results demonstrated that H_2O_2, produced from MAO exposure to tyramine, can readily diffuse through the inner mitochondrial membrane and produce a significant increase in mtDNA single strand breaks (Hauptmann et al., 1996). Because of MAO's proximity to the mitochondrial oxidative phosphorylation system, ROS produced by MAO could conceivably disrupt the ATP-dependent electrochemical and contractile functions of cardiomyocytes, given the heavy reliance on mitochondria for

ATP in these cells (Berman and Hastings, 1999; Kaludercic et al., 2011).

In addition to altering mitochondrial energetics, the byproducts of MAO deamination can wreak havoc on the cell by stimulating apoptotic and hypertrophic pathways, and furthermore may trigger other sites within the cell to release ROS. In myocardium, MAO-derived H_2O_2 at low levels can stimulate hypertrophic and fibroblast proliferative pathways, including ERK1/2 (Sabri et al., 1998) and NFAT 3/4 (Kaludercic et al., 2010) while higher levels lead to apoptotic and necrotic pathways via Bax up-regulation (Bianchi et al., 2005). Aldehyde metabolites may initiate lipid peroxidation which can increase levels of 4-HNE (Kaludercic et al., 2010), inactivate proteasomes (Farout and Friguet, 2006) and the ETS (Choksi et al., 2004) as well. Recent studies have specifically investigated the role of MAO and ROS production in the etiology of cardiac disease since cardiomyocytes are constantly exposed to catecholamines *in vivo*. Kaludercic and colleagues studied the role of MAO-derived ROS in the development of cardiac hypertrophy induced with pressure overload through transverse aortic constriction (TAC). The results of these studies showed that compared to MAO-B null mice, wild-type mice showed a significantly greater increase in left ventricle size, decreased fractional shortening, and increased levels of HNE and fibrosis following TAC, signifying higher ROS formation and lipid peroxidation due to MAO activity (Kaludercic et al., 2014). Similarly, studies in a mouse model of diabetes revealed that MAO-A activity and mitochondrial mass in hearts of diabetic mice were higher than in normo-glycemic mice. The researchers hypothesized that insulin resistance had led to the diminished antioxidant capacity of the cell, in turn leading to more susceptibility to MAO-derived ROS, further illustrating the connection between nutrient overload and diminished antioxidant capacity (Mellor et al., 2010; Manni et al., 2013).

The pathophysiologic involvement of ROS derived from NOX activity in striated muscle remains an area of active investigation. NOX-derived ROS has been shown to be increased and associated with mitochondrial dysfunction and insulin resistance in both heart (Vazquez-Medina et al., 2013) and skeletal muscle (Yokota et al., 2009) of obese rodent models. Other reports clearly implicate myocardial NOX involvement in the development of cardiac hypertrophy and fibrosis in O/T2D (Yamamoto et al., 2008; Fukuda et al., 2010), pressure overload and heart failure (Bendall et al., 2002; Byrne et al., 2003; Heymes et al., 2003; Johar et al., 2006). With myocardial ischemia, however, the role of NOX-derived ROS is ambiguous. Despite evidence that NOX expression and activity is induced in experimental (Meischl et al., 2006) and clinical studies (Krijnen et al., 2003) of myocardial ischemia, myocardial NOX2- and $p47^{PHOX}$-deficient mice exhibit no changes in infarct size compared to wild-type following ischemia/reperfusion injury (Hoffmeyer et al., 2000; Frantz et al., 2006). Recent studies have shed some light on these disparate findings. An intriguing study by Matsushima and co-workers showed that myocardial infarct size/area at risk was substantially higher in mice when both systemic NOX2 and myocardial NOX4 was absent, and they attributed this to a downregulation of adaptive response pathways mediated by hypoxia-inducible factor-1α (HIF1α) in the hearts of these double-knockout mice (Matsushima et al., 2013). Another recent study provided very compelling evidence that NOX4 activity is absolutely necessary for maintaining optimal cardiac redox environment under normoxic conditions, and that eliminating cardiac NOX4 promoted reductive stress and exacerbated ischemia/reperfusion injury in mice (Yu et al., 2014). These studies, and others, illustrate the highly complex roles for NOX in the heart, demanding that further investigation of ROS production is necessary to understand the extent to which perturbations of the redox environment underlie cardio-metabolic diseases.

Until recently, LPPs have been viewed as mere bi- products and bio-markers of oxidative stress (Gutteridge, 1995; Niki, 2009). A rapidly growing body of scientific literature now places LPPs at the front of pathophysiology for a large number of diseases involving striated muscle. In a toxicological study of neonatal cardiomyocytes, HNE caused apoptosis associated with a decrease in GSH, a surge in intracellular Ca^{2+} levels and a loss of mitochondrial membrane potential (Hortigon-Vinagre and Henao, 2014). In skeletal muscle of the diaphragm, protein carbonylation derived from LPPs has been shown to be increased in sepsis, mechanical ventilation and COPD and this contributed to muscle contractile dysfunction in these conditions (Barreiro, 2014).

Several groups have reported increased lipid peroxidation and carbonyl stress in the cardiovascular system of diabetic patients (Arzamastseva et al., 2007; Anderson et al., 2009; Annadurai et al., 2014). Studies in diabetic rat heart show that HNE binds preferentially to cysteine > histidine > lysine residues in the mitochondria (LoPachin et al., 2009). Residues that become modified in this way are often found in the active-site of enzymes, and also in structural motifs responsible for maintaining tertiary and quaternary structure (Korotchkina et al., 2001; Levonen et al., 2004). Mitochondrial protein modification by HNE corresponds to reduced enzyme activity for key enzymes such as adenine nucleotide translocase (ANT), succinate dehydrogenase and α-ketoglutarate dehydrogase in the hearts of diabetic rats (Chen et al., 1995, 2001; Benderdour et al., 2003; Nguyen and Picklo, 2003; Lashin et al., 2006; Chavez et al., 2011). HNE modification of mitochondrial enzymes is represented by yellow lightning bolts shown in **Figure 2**.

COMPENSATORY MECHANISMS FOR ADAPTATION TO LPPs AND CARBONYL STRESS

One of the exquisite features of striated muscle, particularly the heart, is that it is very plastic and can adapt to a broad array of stressors. Carbonyl stress arising from PUFA peroxidation is known to activate genes involved in phase I and II antioxidant/detoxification, mitochondrial biogenesis, and other pathways, all of which serve to restrain the further production of PUFA-derived aldehydes and suppress their toxic effects. A primary adaptive response pathway that is activated by oxidative and carbonyl stress involves NF E2-related factor-2 (Nrf2). Electrophiles such as LPPs and PUFA-derived aldehydes generated during periods of oxidative stress activate Nrf2 by liberating it from its tethering protein Keap1 in the cytosol, allowing it to translocate to the nucleus where it binds to antioxidant/anti-inflammatory response elements (AREs) in promoter regions

of genes/proteins involved in glutathione synthesis and phase II detoxification (Motohashi and Yamamoto, 2004). Studies have shown that HNE induces nuclear accumulation of Nrf2 and up-regulates many GSH-synthesizing enzymes and proteins (reviewed in Chapple et al., 2013). Indeed, pre-conditioning of the heart by retro-orbital injection of HNE induces cardioprotection from ischemia/reperfusion injury via Nrf2, and this cardioprotection is lost in $Nrf2^{-/-}$ mice (Zhang et al., 2010). The roles and regulation of Nrf2, and other transcription factors that activate the ARE, is well beyond the scope of this review, and the reader is referred to other excellent review articles on this topic (Sykiotis and Bohmann, 2010; Tkachev et al., 2011; Chartoumpekis and Kensler, 2013).

In addition to Nrf2, recent evidence has implicated members of the peroxisome proliferator activated receptor (PPAR) family in the cellular response to ROS. Several reports have documented a key role for PPARα and PPARγ in augmenting the antioxidant capacity of liver (Toyama et al., 2004; Abdelmegeed et al., 2009; Guelzim et al., 2011) and vascular tissue (Inoue et al., 2001a,b) in response to oxidized PUFAs. Our group recently completed a small clinical trial and observed that a concentrated dose of fish oil n-3 PUFA's was associated with up-regulation of several antioxidant enzymes, particularly mitochondrial-localized thioredoxin reductase-2 (TxnRd2), and this was accompanied by increased nuclear transactivation of PPARγ, in atrial myocardium of patients undergoing cardiac surgery (Anderson et al., 2014b). This finding is particularly intriguing given the emerging evidence that TxnRd2 is critical for maintaining mitochondrial redox balance in multiple cell types and tissues, including the heart (Rigobello et al., 2005; Stanley et al., 2011; Fisher-Wellman et al., 2013). These articles and other recent literature documenting the beneficial adaptations to ROS in striated muscle under various physiological contexts are listed in **Table 1**.

Another key adaptive response to ROS includes activation of cellular quality control pathways in myocytes, namely autophagy and mitophagy. These pathways are a very active and rapidly expanding area of research, and well beyond the scope of this review. The role of autophagosome formation in the adaptive response to ROS in myocytes are shown in **Figure 2** and is the topic of a number of thorough reviews (Gurusamy and Das, 2009; Lee et al., 2012c; Rahman et al., 2014).

LPP detoxification occurs at two main stages. The first is at the site of lipid peroxidation itself (membrane-localized), and the second is in the cytosol. Following their formation, LPPs may undergo phase I biotransformation where they are conjugated to antioxidant molecules (i.e., glutathione). Alternatively, LPPs are converted by phase II detoxification enzymes such as aldehyde dehydrogenase (Budas et al., 2009), which converts LPPs to their corresponding alcohols. Below we discuss some of the major enzymes involves in the detoxification of LPP-derived aldehydes. It is important to note for many of these enzymes, their enzymatic efficiency is itself decreased by aldehydes which may be key factor in the limitation of antioxidant capacity. A list of the major enzymes responsible for detoxifying LPPs, along with the reaction catalyzed by the enzyme, can be viewed in **Box 1**.

Glutathione-dependent enzymes

Glutathione peroxidase 4. GPX4 is a member of the glutathione peroxide family of selenoenzymes enzymes. In addition to being able to neutralize small hydroperoxides such as H_2O_2, it is one of only a few enzymes capable of neutralizing complex and bulky hydroperoxides (e.g., cholesterol hydroperoxides) (Brigelius-Flohe, 1999; Imai et al., 2003; Yant et al., 2003; Brigelius-Flohe and Maiorino, 2012). GPX4 is unique in that

Box 1 | Mechanism of LPP clearance by different enzyme systems.

Glutathione dependent enzymes

1. Glutathione Peroxidase 4
   - ROOH + 2GSH → GSSG + ROH + H2O
2. Glutathione S-transferase (GST)
   - ROOH + GSH → ROH + GSSG + H_20

Aldehyde Oxidizing enzymes

3. Aldehyde dehydrogenases
   - RCHO + NAD+ + H_2O → RCOOH + NADH + H+

   Molybdenum hydroxylases
4. Aldehyde oxidase (Pryde et al., 2010)
   - aldehyde + H_2O + O_2 → a carboxylic acid + H_2O_2
5. Xanthine Oxidase
   - Xanthine + H_2O + NAD+ ←===→ urate + NADH
   - Hypoxanthine + H2O + NAD+ ←===→ xanthine + NADH
   - Xanthine + H_2O + O2 ←===→ urate + H2O2

Aldehyde reducing enzymes

- Typical reaction: RCHO + NAD+ + H2O → RCOOH + NADH + H+

6. Alcohol reductase
7. Carbonyl reductases
8. Aldo-ketoreductases

Table 1 | Recent literature illustrating beneficial adaptations to ROS in striated muscle.

Muscle	Physiological state/disease	References
Myocardium	Ischemia/reperfusion	Kevin et al., 2003a; Dost et al., 2008; Liu et al., 2008; Zhang et al., 2010; Penna et al., 2013; Tullio et al., 2013; Kalogeris et al., 2014
Myocardium	Exercise	Akita et al., 2007; Sanchez et al., 2008; Nelson et al., 2011; Frasier et al., 2013; Fittipaldi et al., 2014
Myocardium	Nutrient overload	Joyeux-Faure et al., 2006; Anderson et al., 2012; Tocchetti et al., 2012; Fisher-Wellman et al., 2013; Rindler et al., 2013; Chen et al., 2014; Lejay et al., 2014.
Skeletal muscle	Exercise	Gomez-Cabrera et al., 2005, 2008; Kang et al., 2009; Ristow et al., 2009; Powers et al., 2010; Strobel et al., 2011; Powers et al., 2011b; Fisher-Wellman et al., 2013; Radak et al., 2013; Ferraro et al., 2014

unlike other GPXs, it is not limited to glutathione as a substrate (Brigelius-Flohe and Maiorino, 2012). Its protein thiols can perform the function of GSH, and as a result, GPX4 may function as either a glutathione peroxidase or a thiol peroxidase adapting to perform its functions depending on cellular redox conditions.

There are 3 major isoforms of GPX4: cytosolic, mitochondrial and nuclear (Regev-Rudzki and Pines, 2007). In the mitochondria, GPX4 over-expression has been shown to protect against cytochrome c release, generation of H_2O_2 and loss of membrane potential following ischemia/reperfusion injury (Imai et al., 2003). In diabetic mice, over expression of mitochondrial GPX4 was protective against cardiac ischemia reperfusion injury and preserves mitochondrial integrity (Chen et al., 2001; Hollander et al., 2003; Liang et al., 2007; Dabkowski et al., 2008).

Glutathione S-transferase (GST). The GSTs conjugate electrophilic aldehydes with GSH to yield a less reactive conjugate that is eliminated from the cell by glutathione S-conjugate efflux pumps, this process not only increases the solubility of conjugated compounds but marks these structures for elimination from the cell (Hayes and Pulford, 1995). ROS regulate the expression of GST via c- fos and c jun signaling (Daniel, 1993). A study in rat hepatocytes reported that for HNE, metabolism by GST is responsible for the majority of HNE removal (∼60%); a little is metabolized by the alcohol and aldehyde dehydrogenases (10%) leaving ∼24% of 4-HNE clearance for other mechanisms (Hartley et al., 1995).

Aldehyde-metabolizing enzymes:

Aldehyde dehydrogenase. The ALDHs are particularly relevant in the mitochondria as metabolizers of aldehydes generated from PUFAs and monoamine oxidase, as it has been demonstrated that inhibiting ALDH activity has a direct impact on the metabolism of amines by MAO (Ambroziak and Pietruszko, 1991; Rooke et al., 2000). There are 19 known isoforms of the ALDH enzyme with ALDH2 as the major mitochondrial isoform (Marchitti et al., 2008). In human populations, individuals with a polymorphism for ALDH2 had higher levels of the biomarker C-reactive protein following myocardial infarction (Bian et al., 2010). In the SAPPHIRe prospective cohort study, patients with ALDH2 genetic variants with a possessed two-fold greater risk of progression to clinical hypertension (Chang et al., 2012). ALDH3A2 also was found to be important in redox-related pathologies stemming from nutritional overload. It is expressed in both the heart and skeletal muscle and displays a substrate specificity for aldehydes of various long and short chain fatty acids.

Molybdenum hydroxylases. There are two main enzymes in this class (1) Aldehyde oxidase (AOX): This enzyme oxidizes aldehydes to carboxylic acids and it is relatively nonspecific and its endogenous role is not well understood. (2) Xanthine Oxidase: exists as xanthine dehydrogenase-metabolism of aldehydes by xanthine oxidase generates radicals. These two enzymes share some amino acid sequence homology and both contain molybdenum and iron as well as flavin adenine dinucleotide (FAD) as co-factors.

Carbonyl reductases. Carbonyl reductases are part of short chain dehydrogenases/reductases (SDR) family of proteins. These enzymes are ubiquitous and are largely localized to the cytosol with some isoforms in the mitochondria and peroxisomes (Rosemond and Walsh, 2004).

Aldo-ketoreductases. The aldose reductases metabolize lipid aldehydes and their glutathione conjugates (Srivastava et al., 2003). AKR1 specifically metabolizes aldehydes produced by MAO using NADPH. The mitochondrial isoform accounts for 5% of cell activity and metabolizes 4-HNE. AKR1B uses both NADH and NADPH and is also localized in the cytosolic fraction (Srivastava et al., 1999). It is highly expressed in the cardiovascular system, appears to be partly responsible for reducing aldehydes such as 4-HNE, and its activity is potentiated when the substrate is conjugated to glutathione.

ROLE OF ROS IN CARDIAC PRECONDITIONING

The role of ROS in cardiac health and disease has become increasingly recognized in research as a part of normal physiology and pathophysiology due to the multitude of cell regulatory layers associated with redox biology. Although once thought of as strictly deleterious to cardiovascular function, the production of ROS can also elicit an adaptive cardiac preconditioning response. These beneficial ROS are thought to set in motion signaling events that can elicit alterations in gene expression, as well as acute events that can activate proteins in a post-translational manner. In this regard, several stimuli have been shown to evoke cardioprotection in a ROS-dependent manner such as volatile anesthetics, ischemic preconditioning, and pharmacological compounds. A number of major ROS-mediated cellular pathways activated in response to these stimuli are illustrated in **Figure 3**.

ANESTHETIC PRECONDITIONING

Cardiac preconditioning with volatile anesthetics has received a considerable amount of attention as a strategy to mitigate I/R injury through their negative inotropic, anti-arrhythmic and myocardial infarct salvaging effects (Sahlman et al., 1995; Cope et al., 1997; Schlack et al., 1997; Lamberts et al., 2009; Riha et al., 2011; Wojtovich et al., 2011). Several reviews have highlighted mechanisms involved in anesthetic-induced cardioprotection, and ROS have been commonly identified as an important element to the protective mechanism (Hu and Liu, 2009; Bonney et al., 2014; Swyers et al., 2014). ROS are known to be intricately involved in post-ischemic myocardial dysfunction and infarction, and a reduction in oxidative stress has been identified as an important component in alleviating I/R injury; (Hamilton et al., 2004; Dabkowski et al., 2008). Paradoxically, transient bursts of ROS during anesthetic treatment plays an important role in the cardioprotective response (Mullenheim et al., 2002; Novalija et al., 2002; Tanaka et al., 2003). In line with these concepts, treatment with isoflurane (Tanaka et al., 2002, 2003) or sevoflurane (Kevin et al., 2003b) increases ROS production during preconditioning, and reduces oxidative stress during I/R (Nakamura et al., 1999; Kevin et al., 2003b). Following anesthetic exposure, an increase in intracellular superoxide production can be detected by the

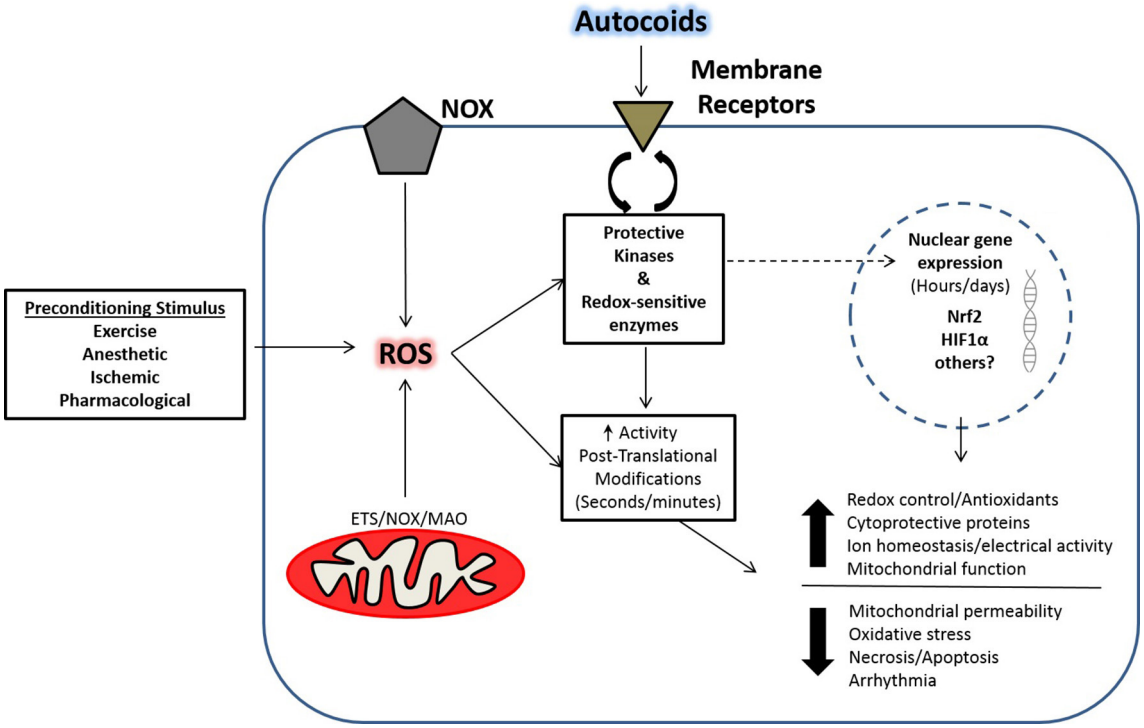

FIGURE 3 | Pre-conditioning and ROS-mediated effects in striated muscle. Shown is a schematic illustrating the development of ROS-mediated adaptations from various preconditioning paradigms as described in the text. Adaptive responses that lead to preconditioning in cardiac muscle culminate in protection through early post-translational modifications to redox sensitive enzymes, and late adaptations through enhanced translocation of key transcription factors. The ensuing adaptations result in the early- and late-window of preconditioning that have been described in the cardioprotection literature. (ROS, Reactive oxygen species; NOX, NADPH-oxidase; ETS, Electron transport system; MAO, Monoamine oxidase; Nrf2, NF E2-related factor-2; HIF1α, Hypoxia-inducible factor-1α; Autocoids, factors secreted from cells with paracrine-like effects).

fluorescence of oxidized dihydroethidium (Tanaka et al., 2002, 2003; Ludwig et al., 2004). This transient ROS burst is important for the preconditioning phenotype as antioxidant treatment negates the beneficial effects. In particular, isoflurane-induced infarct salvage is abolished when bracketing anesthetic administration with intravenous infusion of various antioxidants. These antioxidants include: N-2-mercaptoproprionyl glycine (MPG), Mn(III) tetrakis(4-benzoic acid) porphyrine chloride (MnTBAP), mitotempol, or the glutathione precursor N-acetylcysteine (NAC) (Mullenheim et al., 2002; Tanaka et al., 2003; Hirata et al., 2011). Similarly, the infarct salvaging effect of sevoflurane is abolished by various ROS scavengers such as glutathione, superoxide dismutase, catalase, MPG or MnTBAP (Novalija et al., 2002; Kevin et al., 2003b; Lamberts et al., 2009). Studies comparing desflurane and sevoflurane have demonstrated similar mechanisms of protection during a metabolic insult (Hanouz et al., 2007; Sedlic et al., 2009). *In vitro* studies have also corroborated the importance of ROS in anesthetic preconditioning. One study demonstrated that human right atrial contraction during hypoxia/reoxygenation was better preserved following treatment with desflurane and sevoflurane, and the protective benefits were lost with administration of MPG (Hanouz et al., 2007). Another study demonstrated that desflurane and sevoflurane protected rat isolated left ventricular cells against H_2O_2 induced cell death, and the antioxidant trolox abolished protection. Interestingly, ROS production by desflurane was higher than sevoflurane during preconditioning, and this translated to greater myocyte survival (Sedlic et al., 2009). Taken together these studies provide a compelling argument that transient ROS production acts by influencing downstream cardioprotective signaling events. The mechanism of ROS release as a protective stimuli in anesthetic-induced preconditioning has also been linked to the protection observed with the ischemic preconditioning (IPC) phenomenon, suggesting that these two regimens have commonalities in their cardioprotective mechanisms (Swyers et al., 2014). Indeed IPC also has a strong literature implicating ROS as a triggering mechanism in cardioprotection.

ISCHEMIC PRECONDITIONING

The discovery of IPC by Murry et al., in 1986 has paved the way for an ample amount of research dedicated toward unveiling underlying mechanisms of cardioprotection. IPC is accomplished through short intermittent bouts of I/R, typically one to five cycles, that leads to endogenous protection against an ensuing longer duration ischemia (Murry et al., 1986). Since then, several pathways leading to cardioprotection have been proposed, but a central theme that has been a mainstay in the IPC literature is that ROS play an intricate role in the cardioprotective signaling mechanisms (Shiomi et al., 2013). The beneficial effects of IPC include a reduction in myocardial infarction (Murry et al.,

1986), reperfusion arrhythmias (Shiki and Hearse, 1987), stunning (Bolli et al., 1997), and necrotic and apoptotic cell death (Nakamura et al., 2000). A beneficial role for ROS in IPC is now well-established in the protective signaling cascade. Exposure to a single bout of IPC reduces infarct size, and cardioprotection is abolished when various free-radical scavengers are administered before and/or during IPC (Tanaka et al., 1994; Baines et al., 1997; Dost et al., 2008; Liu et al., 2008). Findings on the time frame of ROS production during preconditioning has been equivocal with evidence that the beneficial ROS are generated during the ischemic phase (Kevin et al., 2003a), and others showing the reperfusion phase (Dost et al., 2008). It has been documented by multiple groups that a significant amount of ROS can be generated by mitochondria during ischemia (Vanden Hoek et al., 1998; Becker et al., 1999; Kevin et al., 2003a), which can be blunted with ETS inhibitors for sites I and III. It is also known that there are overlapping mechanisms involved in IPC (Xin et al., 2012). This is evident by studies demonstrating that free-radical scavengers do not abolish IPC-mediated cardioprotection when multiple preconditioning cycles are used (Iwamoto et al., 1991). The preconditioning mechanism is multifactorial (Yang et al., 2010), so the use of multiple cycles may heighten signaling cascades through other pathways irrespective of ROS. The precise mechanisms on how IPC-induced ROS mediate cardioprotection are still debated, and more research is needed to verify spatial and temporal activation in the cell, as well as end effectors. The use of pharmacological agents that that mimic IPC in a laboratory setting may ultimately foster identification of novel therapeutic approaches.

PHARMACOLOGICAL PRECONDITIONING

There are several pharmacological approaches that have been shown to mimic the preconditioning phenomenon. Diazoxide is a potassium channel activator whose cardioprotective characteristics are thought to derive from a ROS preconditioning stimuli leading to activation of downstream end-effectors (Garlid et al., 1997; Tritto et al., 1997; Pain et al., 2000; Forbes et al., 2001). This is supported by studies where the antioxidants MPG and NAC reduced diazoxide-induced ROS production, and block the recovery of LVDP following global I/R (Forbes et al., 2001). Although cardiomyocytes contain both a mitoK_{ATP} channel and a sarcolemmal K_{ATP} channel, diazoxide and the potassium channel blocker 5-Hydroxydecanoate (5-HD) are thought to have a higher specificity for the mitoK_{ATP} channel (Garlid et al., 1996; Yellon and Downey, 2003). In addition, other potassium channel openers nicorandil and cromakalim have been shown to increase superoxide production in isolated hearts, and the mitoK_{ATP} channel blockers 5-HD and glibenclamide decreased ROS production (Obata and Yamanaka, 2000). Follow up studies show that nicorandil protects against *in vivo* I/R injury in rabbit hearts; and the mitoK_{ATP} channel blocker 5-HD abolished protection, while blockade of the sarcolemmal K_{ATP} channel had no effect (Das and Sarkar, 2003). Importantly, treatment with nicorandil during the reperfusion period did not provide protection, which supports the idea that apriori ROS are setting the stage as a trigger for protective signaling cascades. Others studies investigating the cardioprotective mechanism of acetylcholine and bradykinin demonstrate that an increase in ROS production during treatment is a central component to their infarct sparing effects because the antioxidant MPG abolishes protection (Cohen et al., 2001; Krieg et al., 2004). The authors concluded that agents signaling through G_i proteins share common preconditioning pathways, but adenosine induced protection diverts from this hypothesis, because an adenosine receptor agonist co-administered with MPG or 5-HD did not abolish protection (Cohen et al., 2001). Although adenosine receptor activation can reduce infarct size irrespective of ROS signaling, the downstream preconditioning cascade may be similar in regard to intermediate signaling through mitoK_{ATP} channels (Miura et al., 2000). Statin treatment has also been implicated in cardioprotection through the upregulation of ROS production by mitoK_{ATP} channel activation. The improved post-ischemic contractile function observed with parvastatin was abolished with NAC and 5-HD. The authors concluded that parvastatin leads to the activation of mitoK_{ATP} channels and that downstream activation of PKC mediates the protective phenotype. However, the order of events in this signaling cascade is still under investigation. Mild mitochondrial uncoupling has also been implicated as a preconditioning mechanism, although irrespective of mitoK_{ATP} channel activation (Holmuhamedov et al., 1999). Support for a role of ROS release during uncoupling comes from studies where low concentrations of p-(trifluoromethoxy)phenylhydrazone (FCCP) improved post-ischemic mechanical function. These studies demonstrate that FCCP increased ROS independent of mitoK_{ATP} channel activation. Importantly protection was lost with antioxidant administration, but not with mitoK_{ATP} channel blockers (Brennan et al., 2006). Caveats to studies implicating mitoK_{ATP} channels in preconditioning are the use of diazoxide and 5-HD, which has been shown to have non-specific effects (Hanley et al., 2003; Drose et al., 2006; Li et al., 2010). In addition, there is still uncertainty as to the function and existence of mitoK_{ATP} channels due to the elusiveness of their molecular composition (reviewed in Szabo and Zoratti, 2014). A recent study using a comprehensive proteomics analysis of mitochondrial inner membrane proteins combined with RNA knockdown technique suggested that *KCNJ1*-encoded renal outer medullary potassium channel (ROMK) is a critical pore-forming subunit of the mitoK_{ATP} channel (Foster et al., 2012). Clearly, more studies using targeted molecular approaches such as this are needed to precisely identify all components of this potentially very important channel.

SOURCES OF ROS: WHERE DO THEY COME FROM?

Many of the preconditioning studies reviewed thus far suggest that beneficial ROS production from various stimuli are mitochondrial in origin (Baines et al., 1997; Vanden Hoek et al., 1998; Das et al., 1999; Cohen et al., 2001; Tanaka et al., 2002; Andrukhiv et al., 2006; Sedlic et al., 2009). There has been an ample amount of research showing that the opening of mitochondrial channels/pores and fluctuations in mitochondrial membrane potential leads to the release of ROS (O'Rourke et al., 2007; Aon et al., 2010; Brown and O'Rourke, 2010; Gauthier et al., 2013), but whether channel/pore opening acts as a trigger vs. a mediator of cardioprotection is still being debated. While it is known that ROS are

a commonality to IPC, controversy exists as to when the ROS are released. In isolated chick cardiomyocytes exposed to hypoxic preconditioning an increase in ROS production was observed during the hypoxic period that is abrogated with the complex III inhibitor myxothiazol, but not a NOX or NOS inhibitor (Vanden Hoek et al., 1998). Although ROS have been shown to be released during the ischemic cycle of IPC (Vanden Hoek et al., 1998; Kevin et al., 2003a), Dost et al. propose that ROS generated during the reperfusion phase are providing the redox signals for cardioprotection because MPG administered during the ischemic cycle did not abolish the infarct salvaging effect of IPC (Dost et al., 2008). More recent studies implicate mitochondrial derived ROS, nitric oxide, and the mitochondria redox state as possible regulators of mitoK$_{ATP}$ channel opening in IPC-mediated cardioprotection (Queliconi et al., 2011). Pharmacological activation of the mitoK$_{ATP}$ channel is presumed to proceed in a similar manner to that of IPC, however, as mentioned previously, the relationship between PKC activation, ROS production, and mitoK$_{ATP}$ channel activation is still being investigated (Tritto et al., 1997; Pain et al., 2000; Liu et al., 2008; Yang et al., 2010). The mechanism of ROS release following mitoK$_{ATP}$ channel opening is thought to occur due to mitochondrial uptake of potassium leading to slight alkalization and mild uncoupling (Andrukhiv et al., 2006), however the precise mechanism is unknown. Further evidence using the mitoK$_{ATP}$ channel openers diazoxide or cromakalim suggests that ROS are derived from site I of the ETS because rotenone did not have an effect on myxothiazol-induced ROS production (Andrukhiv et al., 2006). Also, complex III of the ETS has been shown to generate ROS during IPC (Vanden Hoek et al., 1998); and similar findings were reported when mitoK$_{ATP}$ channels were activated through bradykinin-induced preconditioning (Oldenburg et al., 2004). Furthermore, complex III generated ROS appear to also mediate preconditioning with isoflurane because myxothiazol treatment abolished the ROS burst as well as the infarct salvaging effects of isoflurane (Ludwig et al., 2004). Taken together, these findings suggest that mitochondrial derived ROS, particularly from the ETS, are responsible for a large portion of ROS release in models of preconditioning. This further supports the idea of an optimal "zone" where ROS release results in favorable adaptions, whereas exacerbated ROS release can lead to maladaptive responses in diseased states.

END-EFFECTOR MECHANISMS: WHAT LEADS TO PROTECTION?

If ROS are a playing a substantial role in cardioprotection the question becomes, what are the end-effector mechanisms? In models of IPC there are several hypothesis implicating ROS as a means to an end-effector response. An increase in ROS production during IPC is proposed to activate an inner mitochondrial PKC-ε that phosphorylates and inhibits mitochondrial permeability transition (Costa et al., 2006; Costa and Garlid, 2008). A higher threshold for mitochondrial permeability transition following IPC has been demonstrated by several groups and is thought to be one of the mechanisms by which IPC reduces I/R injury (Argaud et al., 2004; Costa et al., 2006; Clarke et al., 2008). Studies have shown that PKC-ε interacts with mitochondrial proteins that constitute the transition pore, and that PKC-ε can phosphorylate the mitochondrial voltage-dependent anion channel (VDAC) *in vitro* (Baines et al., 2003). However, others have failed to observe a change in phosphorylation of mitochondrial permeability transition pore components following IPC (Clarke et al., 2008). This study did observe that IPC inhibited mitochondria permeability transition, but attributed it to a reduction in oxidative stress during I/R injury; although they did not rule out PKC-ε as part of the protective signaling mechanism. Further support for the importance of PKC is provided by studies demonstrating that preconditioning with diazoxide can be blocked when the PKC inhibitor chelerythrine is administered either before diazoxide pretreatment or 10 min before I/R (Takashi et al., 1999), suggesting that the activation of a PKC-ε isoform is playing a downstream role in this form of cardioprotection. However, others hypothesize that PKC activation during IPC sensitizes the myocardium to adenosine, leading to the activation of reperfusion injury salvage kinases such as Akt and ERK (Kuno et al., 2008; Yang et al., 2010).

EXERCISE-DEPENDENT ADAPTATIONS EVOKED BY ROS

The relationship between an active lifestyle through physical activity/exercise with increased longevity and decreased mortality is well known (Hamalainen et al., 1995; Lee et al., 2012a). Although there are numerous adaptive responses following exercise, adaptations at the cellular level present as an increase in skeletal muscle mitochondrial content, which is a hallmark of aerobic exercise training and increased endurance capacity. It has been demonstrated that ROS production is important for this hormetic effect of exercise (Kang et al., 2009), and that co-administration of antioxidants may abolish some of the observed health benefits (Ristow et al., 2009). Therefore, it is of interest to determine what and how adaptations are evoked by exercise-induced ROS.

SOURCES OF ROS DURING EXERCISE

Mechanical loading of cardiac (Sanchez et al., 2008; Prosser et al., 2011) and skeletal (Ihlemann et al., 1999; Chambers et al., 2009) muscle during contraction/relaxation has been implicated as a mechanism by which ROS signaling can evoke adaptive responses. Mechanical stretch of mouse extensor digitorum longus (EDL) muscle increases ROS production and promotes enhanced glucose uptake, independent of insulin signaling, and this is abolished with various ROS scavengers (Chambers et al., 2009). An increase in skeletal muscle ROS production during stretch and contraction is evidenced by increases in the fluorescence of the H_2O_2 sensitive probe, CM-H_2 DCF (Sandstrom et al., 2006; Chambers et al., 2009) and GSSG:GSH$_T$ levels (Sandstrom et al., 2006), neither of which was observed in the presence of the antioxidant NAC. In line with the idea that intracellular ROS production can influence surrounding tissue, one study demonstrated that local ROS production by contracting skeletal muscle may provide a signal to increase the tissue repair response following exercise. NOX-generated H_2O_2 can facilitate the recruitment of neutrophils to skeletal muscle tissue following intense exercise (Nunes-Silva et al., 2014). The manner by which H_2O_2 produced this response was through increased intracellular adhesion molecule expression

leading to enhanced leukocyte-endothelial interaction within the muscular micovasculature endothelial cells. These findings raise the question as to the locus of ROS production, skeletal muscle vs. vascular, since both tissues are known expressers of the NOX2 isoform (Frey et al., 2009; Barbieri and Sestili, 2012).

A similar role for NOX2 in cardiac tissue has been demonstrated whereby myocardial stretch and tachycardia increases NOX2 generated ROS leading to redox modification of the ryanodine receptor and increased calcium spark frequency (Sanchez et al., 2008; Prosser et al., 2011). Further implications for NOX2 derived ROS in cardiac adaptations to exercise was demonstrated by our laboratory, where inhibition of NOX2 abolished the infarct salvage effect of exercise in rats (Frasier et al., 2013). Indeed other sources of ROS production may be mediating adaptations to exercise as well. Some studies suggest that complex I generated ROS and subsequent release into the cytosol through mitoK_{ATP} channels can evoke cardioprotection (Domenech et al., 2002; Andrukhiv et al., 2006), but the evidence for this as a mechanism of ROS production during exercise is scarce. The phospholipase A2-lipoxygenase pathway has been shown to release ROS into the extracellular space in rat skeletal muscle preps, although the physiological implications for this have not been determined (Zuo et al., 2004). Another factor to take into consideration is the degree and spatial production of ROS as a function of exercise intensity.

During high intensity/exhaustive exercise the production of ROS by xanthine oxidase (XO) appears to increase plasma biomarkers oxidative stress (Vina et al., 2000). An increase in plasma XO activity was shown to be important for the activation of adaptive responses to exercise in skeletal muscle (Gomez-Cabrera et al., 2005), because inhibition of XO with allopurinol prevented the activation of MAPKs and abolished the increase in MnSOD gene expression associated with exercise. However, whether or not these effects were due to ROS generated in the plasma vs. the cytoplasm cannot be inferred. Allopurinol may instead be inhibiting the production of intracellular ROS and preventing activation of redox sensitive pathways, which has been shown to be the case for the activation of PGC-1α following acute exercise (Kang et al., 2009). The role of XO on ROS production has been shown to be miniscule in other studies, where allopurinol did not mitigate ROS production as assessed by GSSG levels. Moreover, XO inhibition did not attenuate exercise-induced mitochondrial biogenesis and antioxidant gene expression with chronic exercise (Wadley et al., 2013), indicating that other ROS producing enzymes may be more important for these adaptations to exercise.

With respect to LPPs formed during exercise, measurements of protein carbonylation have yielded surprising and even contradictory results. One recent study reported that chronic exercise in middle aged human male subjects resulted in increased urinary levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), 4-HNE carbonyl adducts, and immune system activation, indicative of a greater level of protein carbonylation (Sasaki et al., 2013). However, another study showed that protein carbonylation was decreased in skeletal muscle of rats following an acute bout of swimming (Magherini et al., 2013). This suggests that LPP formation and subsequent protein carbonylation following exercise is complex, and there may be differences in compartmentalization into blood, muscle, and other organs, in addition to time-dependent factors and type of exercise (i.e., acute vs. chronic) to consider. In any case, these findings illustrate the need for a more detailed investigation of the mechanisms and role of LPPs in exercise.

MECHANISMS RESPONSIBLE FOR ROS-DEPENDENT ADAPTATIONS TO EXERCISE

It is now commonly accepted that ROS have both beneficial and detrimental effects on the cell depending on the delicate balance between scavenging and production. There are several fates of ROS that typically play out in a finite span of time due to their relatively short half-life. While it was originally thought that exercising muscle produces ROS that could be detrimental to the cell through the oxidation of macromolecules, the paradigm has shifted over the last several years. However, the mechanism by which ROS exert beneficial cellular adaptations has been hard to identify, and is an area of research that is dynamically evolving. In addition, the production of ROS, or ROS byproducts, by exercising muscle may lead to direct or indirect alterations to the vasculature, or other organs, in an endocrine like manner. However, whether or not exercise-induced adaptations arise from ROS endocrine-like signaling has not been established. In the literature, there are discrepant findings for changes in blood markers of ROS production following different exercise intensities. Recently Bloomer's group did not observe changes in blood H_2O_2, malondialdehyde (MDA), or advanced-oxidation protein products immediately after an acute aerobic or high intensity exercise bout in trained men (Bloomer et al., 2005). Similar results have been demonstrated after acute exercise in regard to changes in MDA, although elevated levels of ROS were evident post-exercise as measured by electron spin resonance spectroscopy and an increase in the ratio of oxidized glutathione to total glutathione ($GSSG:GSH_T$) (Groussard et al., 2003; Bloomer et al., 2005). These discrepant findings have several implications when taken together: (1) changes in circulating oxidative stress biomarkers may not be indicative of intracellular ROS production; (2) some assays may not be sensitive enough to detect ROS biomarkers; or (3) circulating antioxidant capacity is sufficient to handle the oxidative burden if ROS do get into circulation. While ROS may play an integral role in overall adaptations to exercise, these studies suggest that the beneficial effects of ROS on muscle may be more local rather than exerting a direct effect on systemic adaptations.

Exercise sets in motion adaptive signaling partly through cellular ROS acting as second messengers to alter redox sensitive enzymes (Sadoshima, 2006). Following exercise the cellular redox status transitions toward a more oxidized environment, leading to the activation of endogenous protective mechanisms (Frasier et al., 2011a, 2013). The ROS generated during exercise likely play a major role in the upregulation of antioxidant defense mechanisms, as evidenced by the increase in GR that results after acute exercise. Importantly this up-regulation is abrogated in cardiac muscle when NOX2-generated ROS are blocked prior to exercise (Frasier et al., 2013). ROS also play an important component

in the nuclear translocation of Nrf2 and its binding activity as a transcription factor in cardiac muscle. The importance of this response following 2 days of exercise is demonstrated in Nrf2 knockout mice that display an exacerbated oxidative stress response to exercise with a decrease in key antioxidant enzymes and precursors (Muthusamy et al., 2012). Similarly, in skeletal muscle ROS appear to be important for the activation of transcription factors for mitochondrial biogenesis, as daily vitamin C and E supplementation negated the increase in cytochrome c oxidase subunit IV and PGC-1α following 11 weeks of endurance training in humans (Richters et al., 2011). Further support for *in vivo* local ROS production by the exercising skeletal muscle has been provided by microdialysate samples that were analyzed for cytochrome c reduction as a marker of superoxide production (Hellsten et al., 2007). It has also been shown that skeletal muscle produced ROS may facilitate angiogenesis and increased capillary density through enhanced VEGF production (Kosmidou et al., 2001), but *in vivo* findings with antioxidant supplementation indicate that ROS may not be an important component to the VEGF response from exercise (Hellsten et al., 2007). Evidence for direct ROS-mediated adaptations is difficult pin down due to their promiscuous nature, but the aftermath of an increased oxidized environment is a tell-tale sign of ROS-mediated effects. These studies provide strong evidence for a direct effect of ROS on the enhanced antioxidant response to exercise and indicate this as one of the primary adaptive signaling events in both cardiac and skeletal muscle.

COMPENSATORY CHANGES IN ENDOGENOUS ANTIOXIDANT DEFENSES AND OXIDATIVE PHOSPHORYLATION CAPACITY

In regard to adaptations, ROS production during exercise may act as a prophylactic in the mitigation of a greater oxidative stress, as in myocardial ischemia/reperfusion injury, or skeletal muscle oxidative stress during nutrient overload. It is well documented in the exercise cardioprotection literature that acute exercise improves the redox buffering capacity of the cell as evidenced by an increase in redox specific enzymes such as glutathione reductase (GR), thioredoxin reductase, and MnSOD (Yamashita et al., 1999; Frasier et al., 2011b, 2013; Lee et al., 2012b; Fisher-Wellman et al., 2013). Changes in the activity of H_2O_2 scavenging systems are less robust in the response to exercise, with most studies finding little to no difference in glutathione peroxidase, catalase, and thioredoxin (Frasier et al., 2011a). An enhanced ability to cope with an oxidative stress of greater magnitude is responsible for a large portion of the exercise-induced adaptations afforded by exercise. Several studies have demonstrated that when antioxidants are administered prior to exercise, the protection against myocardial infarction and maintenance of mechanical function following I/R are lost (Yamashita et al., 1999; Akita et al., 2007; Nelson et al., 2011). This implies that ROS are acting through redox circuits during an exercise bout to modify the cellular response to that stressor; and without this signal, some adaptations are lost. However, the locus of ROS production for cardiac adaptions to exercise is still under investigation. We recently found that cytoplasmic ROS generated by NOX2 increases GR activity through post-translational modifications, and the NOX2 inhibitor apocynin abolished the increase in activity immediately after, and 24 h after exercise. While not all studies have shown ROS to be a requirement for cardioprotection (Taylor and Starnes, 2012), it is hard to refute the evidence that there are beneficial ROS that can lead to favorable adaptions.

It is well known that exercise training increases skeletal muscle mitochondrial density, with a subsequent increase in aerobic capacity. But of equal importance is the quality and oxidative phosphorylation capacity of individual mitochondrion. Rats bred for high-capacity running have increased maximal ADP-stimulated respiration and greater complex IV capacity compared to rats bred for low-capacity running (Tweedie et al., 2011). Although the high-capacity runners have higher basal rates of H_2O_2 emission in soleus muscle, they also exhibit a compensatory increase in SOD activity. Furthermore, they had lower DNA damage as assessed by 8-OHdG. These findings are important as a clue to skeletal muscle adaptations to exercise, as enhanced exercise capacity is linked with a favorable compensation to ROS scavenging. Enhanced respiratory capacity as an adaptation to exercise permits cellular energetics to be better maintained, with fewer limitations on the system, and findings in permeabilized fibers from sedentary humans undergoing endurance and strength training programs support this as well. Following endurance and strength training, oxidative phosphorylation capacity increases with the greatest shift in capacity arising from fatty acid oxidation (Pesta et al., 2011). Similar to skeletal muscle, enhancements in cardiac mitochondrial phosphorylation capacity and efficiency was demonstrated in a mouse model of diet-induced obesity and exercise. Isolated cardiac mitochondria from mice exposed to a high-fat diet and 8–10 weeks of high-intensity interval training displayed higher maximal respiratory capacity and ADP phosphorylation to oxygen consumption (P/O) ratios (Hafstad et al., 2013) compared to sedentary counterparts. Importantly, these adaptations resulted in significant improvements in LV function and reductions in myocardial fibrosis. We have also observed compensatory responses to the antioxidant network from exercise and high calorie diets in heart and skeletal muscle, whereby thioredoxin reductase-2 plays an important role in modulating the levels H_2O_2 produced during fatty acid oxidation (Fisher-Wellman et al., 2013). Insight from these studies demonstrates that mitochondrial adaptations/maladaptations are involved in the etiology of cardiometabolic abnormalities, and antioxidant defense mechanisms and respiratory capacity play an important component in affected tissues. In combination, adaptations to these components allows for tighter regulation of redox- and energy-homeostasis during episodes of increased oxidative stress that is encountered with disease pathology.

RECONCILING THE PARADOX: THE ROAD AHEAD

The mechanisms underlying this contrast in outcomes between therapeutically-induced and disease-induced oxidative stress are not clear, as differences in source of ROS, sub-cellular and tissue compartmentalization, temporality of ROS production (i.e., pulsatile vs. constitutive), and transcriptional response pathways are all likely involved to some extent. Specific pathways that have been shown to be involved in mediating the adaptive responses to oxidative stress in striated muscle include activation of ROS scavenging networks, increases in mitochondrial

biogenesis/respiratory capacity, and activation of cell quality control systems (e.g., autophagy/mitophagy). When oxidative stress is prolonged or of high enough magnitude in disease settings, the myocyte still responds in a controlled manner; only this response is a result of a highly oxidized environment that clearly results in a maladaptive phenotype. Overwhelming levels of oxidative stress in disease states leads to alterations in myocyte mitochondrial function and polarity, cell death, and hyper-activation of neutrophils/macrophages with subsequent fibrosis.

Unfortunately the contrast between beneficial ROS and maladaptive ROS may not be as clear cut as originally thought due to the complexity of regulation in biological redox signaling. From a therapeutic standpoint, this ambiguity makes it very difficult to implement antioxidant therapies in the clinic, as there is now very compelling evidence that antioxidants exert negative effects under certain physiological contexts, and in some cases may actually do more harm than good (Ristow, 2014). Moreover, it is now clear that a fine regulation of redox signaling is layered on top of gene regulation and extracellular-regulated responses to oxidative stress. For example, recent findings concerning the role of "mitohormesis" in maintaining homeostasis, particularly under various contexts of nutrient deprivation and overload, serve to add another layer of complexity to the role of redox signaling in the cell (Ristow and Schmeisser, 2014; Yun and Finkel, 2014). Never-the-less, these complexities only serve to underscore the importance of understanding how cellular networks respond to oxidative challenges. If we are to move forward in our comprehension of these networks then there has to be a more rigorous evaluation of the specific intra/intercellular systems that are evoked by ROS, and the key signaling events that are involved. Steps in this direction will further our understanding as to what controls the switch in response to beneficial vs. harmful oxidative stress. Uncovering these regulatory control nodes may 1 day lead to advances that allow us to remain in the Goldilocks Zone en route to dampening the burden of cardio-metabolic disease.

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The impact of age-related dysregulation of the angiotensin system on mitochondrial redox balance

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Aging is associated with the accumulation of various deleterious changes in cells. According to the free radical and mitochondrial theory of aging, mitochondria initiate most of the deleterious changes in aging and govern life span. The failure of mitochondrial reduction-oxidation (redox) homeostasis and the formation of excessive free radicals are tightly linked to dysregulation in the Renin Angiotensin System (RAS). A main rate-controlling step in RAS is renin, an enzyme that hydrolyzes angiotensinogen to generate angiotensin I. Angiotensin I is further converted to Angiotensin II (Ang II) by angiotensin-converting enzyme (ACE). Ang II binds with equal affinity to two main angiotensin receptors—type 1 (AT_1R) and type 2 (AT_2R). The binding of Ang II to AT_1R activates NADPH oxidase, which leads to increased generation of cytoplasmic reactive oxygen species (ROS). This Ang II-AT_1R–NADPH-ROS signal triggers the opening of mitochondrial K_{ATP} channels and mitochondrial ROS production in a positive feedback loop. Furthermore, RAS has been implicated in the decrease of many of ROS scavenging enzymes, thereby leading to detrimental levels of free radicals in the cell. AT_2R is less understood, but evidence supports an anti-oxidative and mitochondria-protective function for AT_2R. The overlap between age related changes in RAS and mitochondria, and the consequences of this overlap on age-related diseases are quite complex. RAS dysregulation has been implicated in many pathological conditions due to its contribution to mitochondrial dysfunction. Decreased age-related, renal and cardiac mitochondrial dysfunction was seen in patients treated with angiotensin receptor blockers. The aim of this review is to: (a) report the most recent information elucidating the role of RAS in mitochondrial redox hemostasis and (b) discuss the effect of age-related activation of RAS on generation of free radicals.

Keywords: mitochondria, angiotensin II, angiotensin II type 1 receptor blockers, mitochondrial angiotensin system, redox regulation, aging, frailty

MITOCHONDRIA AND ANGIOTENSIN SYSTEM: OVERVIEW

It is well accepted that mitochondria are the major source of ATP, which is the fuel for many cellular processes. However, mitochondrial function extends well beyond bioenergetics (Kang and Pervaiz, 2012). Accumulating evidence suggests that mitochondria are also signaling organelles that interact with the rest of the cell through ROS and an array of other signaling complexes (Shigenaga et al., 1994; Turrens, 2003; Chandel, 2014). Earlier studies suggested that mitochondrial ROS were detrimental byproducts associated with several pathological conditions (Turrens, 2003; Chandel, 2014). However, more recent studies suggest that ROS may have fundamental cellular functions—acting as signaling molecules (Chandel, 2014), stabilizing hypoxia inducible factors (HIFs), and inducing gene expression to promote cellular adaptation to low oxygen levels (Chandel et al., 1998, 2000a). ROS also play a role in tumor necrosis factor (TNF) receptor signaling (Chandel et al., 2000b, 2001; Nemoto et al., 2000; Chandel, 2014) and defense against pathogens (Yu et al., 2012).

RAS is a key hormonal pathway that affects virtually every organ. While many of the endocrine (circulating), paracrine (cell-to-different cell), and autocrine (cell-to-same cell) effects of the RAS are believed to be mediated through the canonical extracellular RAS, an independent and differentially regulated intracellular RAS has also been proposed (Robertson and Khairallah, 1971; Inagami et al., 1986, 1988, 1990; Hunt et al., 1992; Sadoshima et al., 1993; Mercure et al., 1998; Vila-Porcile and Corvol, 1998; Cook et al., 2001; Sherrod et al., 2005; Kumar et al., 2007, 2008, 2012; Peters, 2008, 2012; Abadir et al., 2011, 2012). The major components of RAS are (1) angiotensinogen, derived from the liver; (2) renin, derived from the juxtaglomerular cells of afferent arterioles; (3) angiotensin converting enzyme (ACE), a dipeptidyl carboxypeptidase; (4) angiotensin II, and (5) angiotensin II receptors. Angiotensinogen is a globular protein that serves as a substrate for renin, a glycoproteolytic enzyme. The first step of the RAS pathway is conversion of angiotensinogen to angiotensin I by renin. Angiotensin I is then converted to angiotensin II via ACE enzyme (Griendling et al., 1993). At all levels -endocrine,

paracrine, and autocrine—Ang II binds to two main receptor subtypes, AT_1R and AT_2R. Both belong to the G-protein-coupled receptor family but differ in terms of tissue distribution and cell signaling pathways. Ang II binds with equal affinity to AT_1R and AT_2R. These receptors, in turn, activate multiple signal transduction pathways that include signaling molecules like Nitric Oxide ($NO^{\bullet}$), Protein Tyrosine Phosphatases (PTP), and Mitogen-activated protein kinase (MAPK) (Abadir, 2011; Abadir et al., 2012). AT_1R and AT_2R stimulation generally leads to opposing actions (Abadir, 2011; Abadir et al., 2012) summarized in **Table 1**.

Evidence supporting an important role for RAS in mitochondrial function/dysfunction comes from many sources (Cook and Re, 2012; Ellis et al., 2012; Garcia et al., 2012; Gwathmey et al., 2012; Li et al., 2012; Singh et al., 2012; Wangler et al., 2012; Yu et al., 2012; Zaobornyj and Ghafourifar, 2012; Ferder et al., 2013; Sovari et al., 2013). The main function of mitochondria is to generate ATP via the electron transport chain. Electrons are transferred through complex I to complex IV with oxygen as the final electron acceptor. Damaged ETC complexes may no longer accept electrons, leading to generation of ROS (Kregel and Zhang, 2007). AT_1R knock-out mice exhibit a notable phenotype with increased mitochondrial numbers and average lifespan extension exceeding 25% (Benigni et al., 2009). Other clues of the influence of RAS on mitochondria may be gleaned from prior work, which demonstrated that Ang II infusion in rodents induced cardiomyopathy by increasing mitochondrial ROS generation. In these animals, overexpression of catalase specific for mitochondria, but not peroxisomes, protected them against cardiac hypertrophy, fibrosis, and diastolic dysfunction (Dai et al., 2009; Dikalov and Nazarewicz, 2013). The recent identification of a functional intra-mitochondrial angiotensin system (MAS) provided additional insight into the RAS interface with mitochondria (Eto et al., 2002; Kumar et al., 2008; Abadir et al., 2011, 2012). Recently, changes in MAS and a novel role for AT_1R on mitochondrial respiration in diabetes were reported (Persson et al., in press). In mitochondria from renal tubular cells, expression of AT_2R decreases while AT_1R increases with age. Chronic administration of losartan, an angiotensin receptor blocker, prevented age-related decrease of mitochondrial AT_2R. Other parts of the system including renin and ACE have also been localized intracellularly, with evidence suggesting their presence in the nucleus and mitochondria (Vidotti et al., 2004; Abadir et al., 2012). Interestingly, this intracellular system is independent of circulating RAS; ACE inhibitors fail to block intracellular ACE (Cristovam et al., 2008).

Table 1 | Opposing functions of AT1R and AT2R.

AT1R	AT2R
Vasoconstriction	Vasodilation
↑ cell growth	↓ cell growth
Cellular proliferation	Cellular differentiation
Anti-naturetic	Naturetic
Production of O_2	Production of NO
↑ fibroblast proliferation/collagen synthesis	↓ fibroblast proliferation
Pro-apoptotic	Anti-apoptotic

Several groups demonstrated a tight link between RAS, mitochondria, and a host of age-related pathologic conditions (Inagami, 2011; Carey, 2012; Conti et al., 2012; Cook and Re, 2012; Dai et al., 2012; Ellis et al., 2012; Gao et al., 2012; Garcia et al., 2012; Gwathmey et al., 2012; Horan et al., 2012; Li et al., 2012; Singh et al., 2012; Wangler et al., 2012; Yu et al., 2012; Zaobornyj and Ghafourifar, 2012). In addition, RAS dysregulation aggravates several acute and chronic diseases, many of which have been linked to mitochondrial dysfunction [atherosclerosis (Warnholtz et al., 1999), kidney disease (Ma et al., 1998), myocardial damage after infarction (Kuno et al., 2002), cerebral infarct size after ischemia (Panahpour and Dehghani, 2012)]. Locally activated RAS in heart tissue has been implicated in cardiac hypertrophy and fibrosis (Kumar et al., 2008). The mechanism by which intracellular angiotensin II (iAng II) affects cardiac tissue has been a topic of debate for years. One possible mechanism is that iAng II interacts with intracellular AT_1R or AT_1-like receptors to bring about the observed changes. Another mechanism involves iAng II binding directly to chromatin to promote the transcription of growth factors like insulin, PDGF, and FGF-2 (Baker et al., 2004). iAng II may also may also affect Ca^{+2} fluxes and activate phospholipase C and PKC by binding to AT_1R on sarcolemma, mitochondria, or internalized receptors (Eto et al., 2002; Baker et al., 2004).

Similar to extracellular RAS, intracellular RAS has been implicated in many pathological conditions as well. Intracellularly, RAS is highly active in producing increased amounts of iAng II in mice with advanced heart failure. Increased ventricular hypertrophy or fibrosis was also observed (De Mello and Gerena, 2008). The mechanism by which Ang II is produced has not been defined yet. Pressure overload and mechanical stretch of cardiomyocytes after myocardial infarction may cause secretion of local Ang II. After examination of levels of expression of angiotensinogen, renin, ACE and AT_1 genes, stretched cardiac myocytes were observed to have higher levels of mRNA and RAS enzymes than unstretched myocytes (Malhotra et al., 1999). This increased Ang II seems to work through a mineralocorticoid receptor because the administration of an aldosterone receptor antagonist mitigated the effect of Ang II on inward Ca^{+2} current in failing hearts (De Mello and Gerena, 2008). Ang II further caused cell swelling in failing cardiac myocytes via activation of ionic channels and decrease in gap junction permeability. These molecular changes may lead to altered gene expression, which could be contributing to cardiac remodeling. In addition, changes in ionic and gap junction permeability can decrease action potential duration and conduction velocity. All of these factors may lead to cardiac arrhythmias by causing electrical uncoupling, mechanical desynchronization, and cardiac remodeling (De Mello and Frohlich, 2011). Furthermore, diabetic patients seem to have upregulated intracellular RAS activity because high glucose in rat mesangial cells resulted in ∼30-fold increase in intracellular renin (iRenin) activity and increased iAng II concentrations localized mostly to the nucleus. Localization of iAng II to the nucleus suggests that the mechanism of action of iAng II is both cytoplasmic and nuclear. Renin and chymase (an alternative Ang II-generating

enzyme), were implicated in the glucose-induced increase in Ang II rather than ACE; this confirms that the increased Ang II is not due to increased uptake of circulating Ang II but rather due to localized tissue synthesis (Re et al., 1984; Vidotti et al., 2004; Kumar et al., 2007).

MITOCHONDRIAL REDUCTION-OXIDATION (REDOX) BALANCE: ROLE OF RAS IN ROS GENERATION, TRANSPORT AND ELIMINATION

Mitochondria play a critical role in redox chemistry. Mitochondrial redox balance is the process by which, under physiological conditions, mitochondria maintain a dynamic balance between ROS generation, their transport, and an array of antioxidant systems (glutathione, glutathione peroxidase, glutathione reductase, MnSOD, catalase, and thioredoxin system) in response to fluctuations in cellular energy demand (Aon et al., 2010; Cortassa et al., 2014).

ROS GENERATION AND TRANSPORT

ROS are generated from various sources including NADPH oxidase (NOX2 and NOX4), uncoupled nitric oxide synthase (NOS), xanthine oxidase (XO), and mitochondria. Of these, mitochondria are the main source of ROS (Nickel et al., 2014).

ROS are generated in the respiratory chain, mainly at the level of complex I and III (Murphy, 2009; Kembro et al., 2014) although recent evidence also involves complex II (Drose, 2013).

Electron transfer between the respiratory complexes in the respiratory chain generates a proton motive force composed of proton and electrical gradients that then drives ATP synthesis at the level of ATP synthase. NADH and $FADH_2$, generated in the Tricarboxylic Acid (TCA) cycle act as electron donors for the electron transport chain (ETC) (Rich and Marechal, 2010). Oxygen is the final acceptor of four electrons transferred from the ETC, and converted to H_2O (Mitchell, 1961; Liu et al., 2002). If less than four electrons are transferred to oxygen, ROS are produced (Kregel and Zhang, 2007). Damaged ETC complexes may no longer accept electrons. Excess ROS can damage respiratory complexes and initiate a vicious cycle of ROS overflow thus highlighting the fact that mitochondria can be both source and victim of oxidative stress (Daiber, 2010).

ADP and Ca^{+2}, can modulate the rate of ATP generation according to energy demand. ADP stimulates ATP synthesis via F1F0 ATPase, driven mainly by the electrical component of the proton motive force (Wood, 2006). The dissipation of the mitochondrial membrane potential generates a "pull" of electrons from NADH at the level of complex I, or FADH2 from succinate at the level of complex II thus increasing O_2 consumption.

As a matter of fact, an increase in energy demand, e.g., higher cardiac workload under exercising conditions, will increase both ADP and Ca^{2+} uptake by the mitochondria to increase ATP supply to match the demand (Cortassa et al., 2006; Murphy, 2009). Energized mitochondria will exhibit higher levels of ATP and NAD(P)H and lower electron flow thus increasing the probability of $O_2^{\cdot-}$ generation in the respiratory chain. Consequently, mitochondrial ROS production is highly dependent on the energetic and redox status of mitochondria (Kang and Pervaiz, 2012; Cortassa et al., 2014).

RAS-INDUCED MITOCHONDRIAL ROS GENERATION

As mentioned before, angiotensin II can bind to two major receptors: AT_1R and AT_2R. Ang II binding to AT_1R in the plasma membrane has been implicated in increased ROS production (**Figure 1**). Ang II-AT_1R can activate NADPH oxidase, leading to increased generation of cytoplasmic ROS. This Ang II-AT_1R–NADPH-ROS signal triggers the opening of mitochondrial K_{ATP} (mtK_{ATP}) channels (**Figure 2**) that in turn activates mitochondrial ROS production in a positive feedback loop (Daiber, 2010). Opening of mtK_{ATP} channels decreases mitochondrial membrane potential. This triggers the opening of mitochondrial permeability transition (MPT) channel. The loss of membrane potential due to opening of the inner membrane anion channel (IMAC) (Aon et al., 2003, 2007) or the permeability transition pore (PTP) (Zorov et al., 2000) can produce a burst of mitochondrial ROS leading to ROS-induced ROS release (Zorov et al., 2000; Aon et al., 2003; Zhang et al., 2007).

MAJOR SITES OF $O_2^{\cdot-}$ GENERATION

(i) Complex I or NADH: ubiquinone oxidoreductase is considered one of the major source of superoxide production in the ETC complexes (Brand et al., 2004). Electrons from succinate can flow in reverse to complex I to reduce NAD^+ to NADH. This reverse electron transfer (RET) increases ROS production at the level of complex I. When treated with ADP or malonate, both of which block RET, mitochondrial ROS emission decreases (Liu et al., 2002). Which component of complex I—FMN, Fe-S clusters, or ubiquinone—is producing superoxide is not clear (Brand et al., 2004). Ang II has been shown to damage complex I, thus contributing to

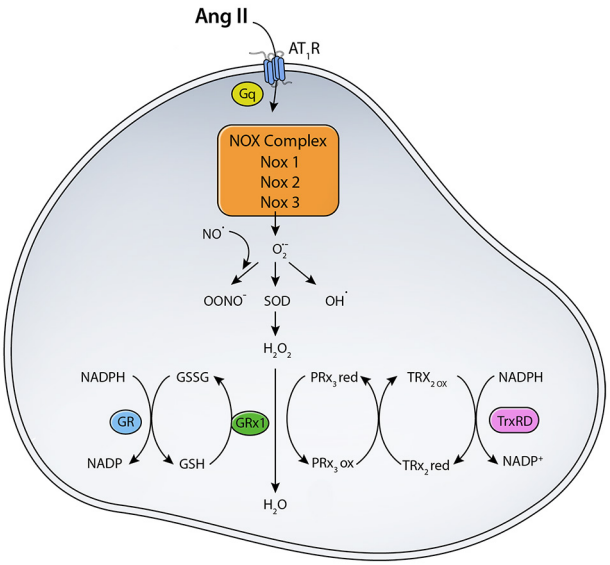

FIGURE 1 | Scheme for RAS induced ROS generation. The binding of Ang II to AT_1R activates NADPH oxidase that transfers an electron from NADPH to O_2generating $O_2^{\cdot-}$. Location and expression level of different NOX enzymes determine their function. (SOD, Superoxide dismutase; TrxRD, thioredoxin reductase; GR, Glutathione Reductase; GPX, Glutathione peroxidase; PRx3, peroxiredoxin 3; TRx2, thioredoxin).

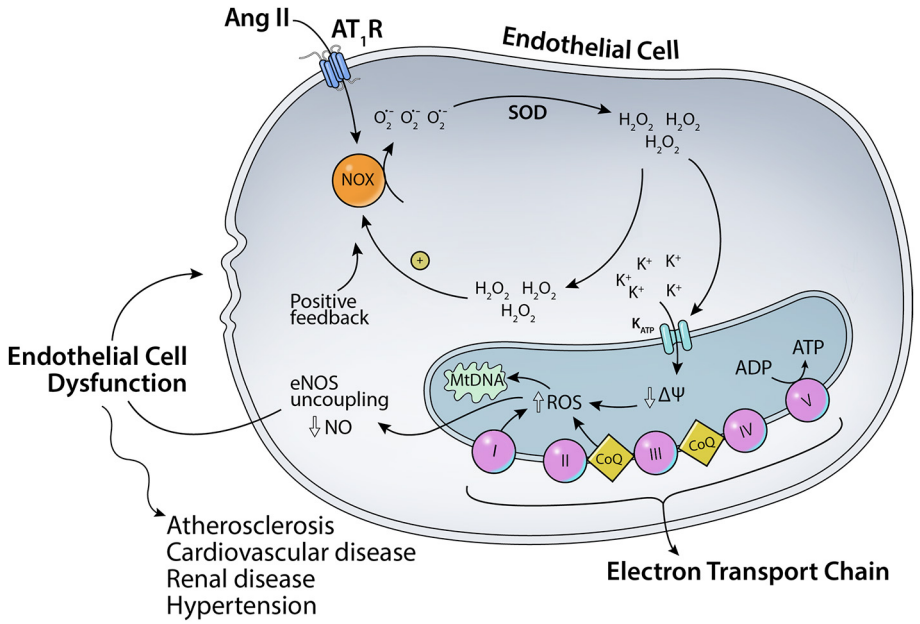

FIGURE 2 | Scheme for the effects of Ang II on mitochondrial K_{ATP} channels. Ang II-AT_1R–NADPH-ROS signal triggers the opening of mitochondrial K_{ATP} channels and mitochondrial ROS production in a positive feedback loop.

ROS generation. Specifically, Ang II is able to down-regulate ND5, a component of complex I, via oxidation of mtDNA thus decreasing electron flow and the likelihood of ROS generation (Ricci et al., 2005).

(ii) Cytochrome b_{c1} complex or complex III is another important source of $O_2^{\bullet-}$ generation. Coenzyme Q, or ubiquinol (QH2), is the electron donor for complex III. Reduced coenzyme Q (QH2) diffuses to the inner side of mitochondrial membrane to the Q_o site, where it transfers one electron to cytochrome c bound to complex III. Cytochrome c then transfers electrons to complex IV. Another QH2 transfers an electron to the oxidized coenzyme Q (Q), reducing it to QH2 near the matrix side of the membrane (Qi site) (Quinlan et al., 2011). After losing an electron to cytochrome c and prior to transferring an electron to Q, ubiquinol may form the intermediate ubisemiquinone (Q.). Electrons can leak (Turrens, 2003) from Q· to oxygen, forming $O_2^{\bullet-}$. Antimycin, a Qi site inhibitor, elicits large amounts of $O_2^{\bullet-}$ when O_2 reacts with ubisemiquinone bound to the Q_o site (Murphy, 2009). Under normal conditions, $O_2^{\bullet-}$ generation from complex I and III varies according to tissue conditions.... For example, complex III appears to be the primary source of $O_2^{\bullet-}$ in heart and lung mitochondria whereas complex I is the major source in the brain (Turrens and Boveris, 1980; Turrens et al., 1982; Barja and Herrero, 1998; Turrens, 2003). In general, acute and chronic infusion of Ang II has been shown to decrease expression of electron transport chain proteins (Larkin et al., 2004). Excessive ROS production due to Ang II can impair complex I and III activities, increasing electron leakage (Prathapan et al., 2014). More information is needed to delineate the role of RAS in the modulation of complex III.

(iii) NADPH oxidase (NOX) family proteins: NOX is a family of transmembrane proteins that may be source of ROS. NADPH oxidase works as a nonspecific host defense system by releasing large amounts of ROS during infections (Thrasher et al., 1994). When cytoplasmic NADPH oxidase is activated, it moves toward membrane-associated cytochrome b558 to form a complex. Cytochrome b558 regulates the enzymatic activity of NADPH oxidase by transferring one electron to molecular oxygen, which gets reduced to $O_2^{\bullet-}$ (Bayraktutan et al., 1998). While neutrophil NADPH oxidase produces ROS in bursts, vascular NADPH oxidases produce low levels of $O_2^{\bullet-}$ continuously (Cai et al., 2003). NADPH-derived cytoplasmic ROS can mediate mtKATP opening enabling K+ influx that produces membrane depolarization and alkalinization of the matrix (Di et al., 2007). This matrix alkalization has been shown to increase H_2O_2 in the presence of an mtKATP opener (Pain et al., 2000; Heinzel et al., 2005; Andrukhiv et al., 2006; Daiber, 2010).

Location and expression level of the different NOX enzymes determine their function. NOX1 is abundant in colon epithelium and has been reported to play a role in host defense in intestinal crypts and on luminal surface (Szanto et al., 2005). NOX2 is expressed in granulocytes and monocyte/macrophages. ROS generation through NOX2 activation has been shown to play a role in killing microbes and inactivating microbial virulent factors. The exact localization and level of expression of the different NOX enzymes are still not completely sorted out yet. NOX3 has been generally located in the cochlear and vestibular sensory epithelia, and spiral ganglion. NOX4 has been shown to be expressed in the kidney and suggested that it plays a role in protection of the vasculature during inflammatory stress. ROS

generation through NOX4 has also been shown to induce HIF-1α transcription factors, which in turn leads to activation of erythropoietin gene in the kidney (Bedard and Krause, 2007; Jung et al., 2008; Schroder et al., 2012). NOX5 is expressed in various tissues including spleen, lymph nodes, and vascular smooth muscle cells (Bedard and Krause, 2007). NOX5 increases Jak2 phosphorylation, which increases vascular smooth muscle cell (VSMC) proliferation in human coronary arteries, aorta, and splenic vessels (Fulton, 2009).

NADPH oxidase is a major source of angiotensin II-induced ROS generation. NOX1 appears to have various roles in VSMCs, including Ang II-induced hypertrophy, serum-induced proliferation, and basic fibroblast-induced growth factor migration (Redmond and Cahill, 2012). It localizes in VSMCs from large arteries (Nguyen Dinh et al., 2013). NOX1 is needed under various physiologic conditions such as thrombin-induced migration, endothelial cell proliferation, cell growth, and vessel formation (Sunggip et al., 2013). Overproduction of $O_2^{\cdot-}$ from NOX1 and NOX 2 can cause renin release, smooth muscle cell proliferation, and decreased $NO^{\cdot}$, all of which lead to endothelial dysfunction and increased sympathetic tone that in turn cause hypertension (Takac et al., 2012). Ang II can signal via phospholipase D and this pathway may cause VSMC proliferation and contractility through NADPH oxidase (Touyz and Berry, 2002). NOX1 distributes in the plasma membrane, primarily generating $O_2^{\cdot-}$ anions (Dikalova et al., 2005; Valente et al., 2012). Ang II activates NOX1 via NOX1-AT_1R interaction. This is supported by a study showing that continuous infusion of Ang II was correlated with increased NOX1 mRNA expression (Valente et al., 2012). NOX1 functions are mostly growth-promoting being highly expressed in proliferating cells. It activates VEGF and matrix metalloproteinase, which promote angiogenesis (Lassegue et al., 2001; Wilkinson-Berka et al., 2014). NOX1 is also responsible for $O_2^{\cdot-}$ generation and is implicated in many pathological conditions including atherosclerosis, diabetes and hypertension (Chose et al., 2008; Briones et al., 2011).

NOX4 is found in subcellular compartments such as the nucleus, endoplasmic reticulum, and mitochondria (Wingler et al., 2001; Briones et al., 2011). Unlike NOX1, NOX4 typically generates H_2O_2 (Briones et al., 2011; Valente et al., 2012). NOX4 serves as an oxygen sensor and regulates erythropoietin synthesis in the kidneys (Geiszt et al., 2000). In VSMCs, NOX4 is found in focal adhesions and shown to maintain differentiation of cells whereas NOX 1 induces growth and proliferation. This phenomenon was seen in multiple tissues. For example, in coronary arteries, NOX4 expression was correlated with α-actin levels in osteoclasts, and over-expressed NOX4 was associated with increased osteoclast markers. NOX4 is important for maintaining physiologic function of many tissues (Clempus et al., 2007). NOX4 is not up- but instead down-regulated when treated with Ang II (Lassegue et al., 2001; Wingler et al., 2001). Both NOX2 and NOX4 are present in the aortic vascular smooth muscle. When infused with Ang II, NOX2 knock-out mice did not have significant hypertrophy. Therefore, NOX2 was deemed essential for Ang II-induced cardiac hypertrophy and NOX 4 did not seem to play a big role in Ang II-induced cardiac pathologies (Byrne et al., 2003). This argument is supported by many research groups that studied Ang II's effects on ROS generation. Interestingly, Ang II doubled the ratio of $O_2^{\cdot-}$ to H_2O_2 ratio in quiescent cells and almost quadrupled it in proliferating cells. In addition, NOX4 is a source of H_2O_2in a variety of vascular cells including fibroblasts, endothelium, and smooth muscles. This constant generation of H_2O_2 might be essential to maintaining cell functions in angiogenesis during wound healing (Dikalov et al., 2008). Increased NOX4 expression is essential in tissue survival during ischemic stress, tissue remodeling, and regulation of glutathione. Interestingly, NOX4 has been implicated in cardiac hypertrophy, interstitial fibrosis, and apoptosis in cardiac muscle; however, cardiac function was still maintained (Zhang et al., 2010; Brewer et al., 2011; Schroder et al., 2012; Sunggip et al., 2013). Though some studies show NOX4 playing a role in pathophysiology, the great majority shows that the physiological role of this enzyme is more relevant.

ROLE OF RAS IN ROS SCAVENGING

To maintain ROS balance, the production of ROS has to be matched with a ROS scavenging mechanisms (**Figure 1**). MnSOD in the mitochondrial matrix converts $O_2^{\cdot-}$ to H_2O_2, which can diffuse across membranes. $O_2^{\cdot-}$ dismutation to H_2O_2 is important for signaling and avoiding oxidative damage, in case of oxidative stress (Kang and Pervaiz, 2012). H_2O_2 is scavenged by glutathione peroxidase (GPX) and peroxiredoxin (PRX). NADPH generated from mitochondrial transhydrogenase (Hoek and Rydstrom, 1988; Jackson, 2003) is the main electron of the GSH and Trx systems (Nickel et al., 2014). Under normal function, GSH and Trx mitochondrial systems are mainly responsible for offsetting most of the ROS produced by the respiratory chain, especially under state 3 respiration when electron flow is maximal (Aon et al., 2012). Other antioxidants localized to cytoplasmic and intermembrane space include Copper-Zinc superoxide dismutase (Cu-ZnSOD) and catalase (Aon et al., 2012). ROS can mediate increased expression of antioxidant enzymes via activation of the Nrf2-antioxidant response element signaling pathway (Nguyen et al., 2009). ROS-activated Nrf2 increases ARE-mediated gene expression, which includes many antioxidants such as glutathione S-transferase, quinine reductase, and heme oxygenase 1 (Bergelson et al., 1994; Alam et al., 1999). Levels of antioxidants are often decreased in disease states or under oxidative stress. For example, diabetes and insulin resistance are correlated with decreased antioxidant capacity due to decreased SOD and glutathione reductase activities (Aon et al., 2012). Under normal conditions, however, antioxidant enzymes play a major role in scavenging ROS generated from various sources like NADPH oxidase, NOS, xanthine oxidase, and mitochondrial electron transport chain enzymes.

Angiotensin II has been implicated in decreasing the activity of scavenging enzymes, thereby leading to detrimental levels of ROS. Ethanol ingestion is associated with oxidative stress and decreased GSH levels via activation of the RAS. When ethanol-fed rats were treated with the AT_1R blocker losartan, GSH levels were maintained (Bechara et al., 2005). SOD are also targets of Ang II. Mammals possess three SOD isoforms, Cu-Zn SOD (SOD1), Mn SOD (SOD2), and extracellular SOD (SOD3) located in the cytoplasm, mitochondria and the extracellular space, respectively

(Rodriguez-Iturbe et al., 2007). SOD1 is a major defense system against Ang II-triggered ROS in the kidneys. SOD1 knockout mice showed 4-fold increase in afferent arteriolar $O_2^{\cdot-}$ levels when treated with Ang II (Carlstrom et al., 2010). In addition, in coronary artery disease patients, SOD3 levels were severely reduced in the human arterial walls and this decrease was associated with oxidative stress and reduced $NO^{\cdot}$ bioavailability; these conditions were shown to contribute to the pathophysiology of the disease (Landmesser et al., 2007). When treated with the AT_1R blocker losartan, an increase in $NO^{\cdot}$ bioavailability and SOD3 activity were seen. A more than 200% increase in SOD3 activity was associated with reduced oxidative stress and improved endothelial function (Hornig et al., 2001). Catalase activity was diminished in Ang II-stimulated cardiomyocytes whereas ROS-elicited by Ang II in mesangial cells implied reduced catalase transcription (Venkatesan et al., 2007; Murtaza et al., 2008; Tan et al., 2008). Reduced catalase mRNA expression and protein levels were noted with Ang II treatment of VSMCs (Xiong et al., 2010). There is also some evidence about the modulation of the Trx system by the RAS system; ACE inhibitors have been shown to improve myocarditis via a mechanism involving the Trx system (Tanito et al., 2004; Touyz, 2004). The RAS not only contributes directly to ROS generation, but also it affects indirectly the redox balance via modulation of various antioxidant enzymes. Treatment methods involving inhibition of RAS enzymes, specifically Ang II type 1 receptor blocker, may be crucial in impeding pathophysiological behavior.

ROS AS SIGNALING MOLECULES

Angiotensin II interacts with various tissues including vascular, renal, and neuronal to induce numerous physiologic signaling cascades and functions. Griendling et al. discussed the role of Ang II in modulating growth-related signaling pathways via ROS signaling (Griendling and Ushio-Fukai, 2000). ROS-mediated oxidation can alter gene expression thorough signaling cascades induction, or interaction with transcription factors. H_2O_2 can reversibly inhibit tyrosine phosphatase PTP1B, a regulator of the insulin signaling pathway (Apel and Hirt, 2004; Combs, 2010). Recent research focuses on PTP1B inhibitors as potential therapeutic treatment for type 2 diabetes and obesity (Thareja et al., 2012). ROS also play a role in tyrosine phosphorylation as shown in platelet-derived growth factor where a transient increase in ROS inactivated tyrosine phosphatase (Finkel, 1998). Nontoxic amounts of H_2O_2 administered extracellularly have been shown to stimulate mitogen activated protein kinase (MAPK), that directly affects the inflammatory response, cell proliferation, differentiation, and survival in response to extracellular stimuli (Stevenson et al., 1994; Arbabi and Maier, 2002). Exogenous H_2O_2 has also been shown to activate JNK, (Finkel, 1998) p38, (Hensley et al., 2000) and NF-$_K$B, a protein complex that induces expression of protective genes during inflammation and infection (Baeuerle and Henkel, 1994).

ROS participate in the host innate immune response. Immune cells undergo respiratory burst, producing high levels of free oxygen radicals during infection, a response that results toxic to pathogens (Spooner and Yilmaz, 2011). When a pathogen invades a cell, Leucine-Rich Repeat-containing family member receptor, NLRX1, moves to the mitochondria to stimulate the electron transport chain and initiate ROS production (Arnoult et al., 2009). Since ROS are produced by NADPH oxidases in mature phagocytic cells, many bacteria, like *Francisellatularensis*, interfere with NADPH oxidase assembly (Cirillo et al., 2009). Oxidative burst within macrophages has been shown to decrease urogenital infections associated with pathogen *Chlamydia trachomatis* (Boncompain et al., 2010).

In addition to fighting infections, ROS have also been implicated in cancer therapy since increased ROS levels may be lethal for tumor cells triggering apoptosis. Direct exposure of cancer cells to ROS generating agents like arsenic trioxide, or alternatively inhibiting antioxidant enzymes with2-methoxyestradiol, a SOD inhibitor, may trigger apoptosis in human leukemia (Zhou et al., 2003; Pelicano et al., 2004).

Overall, available evidence indicates that controlled levels of ROS represent effective signaling molecules, intervening in different cellular processes as diverse as communication, inflammation, immunity, and as therapeutic cancer agents. The role of RAS induced ROS in signaling pathways is discussed next.

EPIDERMAL GROWTH FACTOR (EGF) AND EXTRACELLULAR SIGNAL-REGULATED KINASE 1/2 (ERK1/2)

EGF is required for Ang II effects on various tyrosine kinases, and its activation is initiated by NOX-induced ROS (Eguchi et al., 1998; Griendling and Ushio-Fukai, 2000; Ushio-Fukai et al., 2001). C-Src, a tyrosine kinase activated by Ang II, promotes multiple signaling events including various MAPKs. Ang II-induced activation of c-Src is redox-sensitive since its stimulation is inhibited by antioxidants (Abe et al., 1997; Ushio-Fukai et al., 2001). EGF receptor activation is needed for Ang II action on ERK1/2, a type of MAPK (Eguchi et al., 1996; Liao et al., 1997; Griendling and Ushio-Fukai, 2000). ERK1/2 has an important role in cell adhesion, cell cycle progression, migration, survival, differentiation, metabolism, and proliferation (Roskoski, 2012). Activation of ERK1/2 is said to be redox-sensitive or redox-insensitive in some studies (Sundaresan et al., 1995; Viedt et al., 2000).

MAPK AND ADENILATE KINASE (Akt)

MAPKs control various cellular activities like growth, apoptosis, and stress signals. Four main MAPKs are ERK1/2, c-Jun N terminal kinase (JNK), p38MAPKs, and big MAPK-1. Ang II can activate JNK, ERK1/2, and p38MAPK. Though there is an ambiguity as to whether ERK 1/2 is redox-sensitive or not, p38MAPK and JNK have been shown to be redox-sensitive when activated by Ang II. Ang II triggers ROS generation through NADPH oxidase activation, followed by JNK and p39MAPK stimulation (Griendling and Ushio-Fukai, 2000; Viedt et al., 2000). In VSMCs, Ang II activates Akt, a serine threonine kinase that plays a role in cell survival and protein synthesis. NADPH-derived ROS induces Akt which is associated with heat shock protein 27, also activated by H_2O_2 (Konishi et al., 1997; Coffer et al., 1998; Ushio-Fukai et al., 1999; Griendling and Ushio-Fukai, 2000).

NITRIC OXIDE SYNTHASE (NOS)

NOS is another enzyme capable of producing $O_2^{\cdot-}$ using tetrahydrobiopterin as a cofactor. Superoxide generated from NADPH

oxidases can give rise to peroxynitrite ($ONOO^-$) (**Figure 1**). $ONOO^-$ contributes to oxidation of tetrahydrobiopterin, leading to NOS uncoupling and more $O_2^{\bullet-}$ generation (Landmesser et al., 2003). A mitochondrial variant of NOS (mtNOS) can generate nitric oxide ($NO^\bullet$), a reactive nitrogen species (RNS). Mitochondrial respiration can be partially inhibited by $NO^\bullet$, through inactivation of cytochrome c oxidase from complex IV that can lead to increased ROS production and a vicious cycle of mitochondrial damage by ROS excess (Kang and Pervaiz, 2012). Excess $NO^\bullet$ can react with $O_2^{\bullet-}$ to form the highly reactive $ONOO^-$ which, like $O_2^{\bullet-}$, is unable to permeate the mitochondrial membrane, can cause oxidative damage, nitration and nitrosation (Squadrito and Pryor, 1995; Kang and Pervaiz, 2012). MnSOD can be nitrated by $ONOO^-$, decreasing its activity (Quijano et al., 2001). Ang II-treated endothelial cells released more H_2O_2, and exhibited mitochondrial loss of membrane potential, respiration impairment, and decreased GSH and $NO^\bullet$ formation (Doughan et al., 2008; Daiber, 2010). The reduction of dihydrobiopterin to tetrahydrobiopterin is catalyzed by dihydrofolate kinase. Tetrahydrobiopterin is needed by eNOS for basal $NO^\bullet$ production. Ang II can lead to eNOS uncoupling via H_2O_2 production in a NOX-dependent manner. This results in down-regulation of dihydrobiopterin kinase and diminished tetrahydrobiopterin cofactor and nNOS uncoupling. Dysfunctional eNOS results in decreased $NO^\bullet$ bioavailability (Cai and Harrison, 2000; Sunggip et al., 2013). Ang II-induced $O_2^{\bullet-}$ can react with endothelial $NO^\bullet$ produced by eNOS, decreasing its concentration even more. Loss of $NO^\bullet$ contributes to endothelial dysfunction initiating atherosclerosis (Nickenig and Harrison, 2002). In addition, expression of inflammatory molecules like MCP-1 and VCAM-1 appears to be indirectly regulated by Ang II, accelerating atherosclerosis (Nickenig and Harrison, 2002). Angiotensin II-induced phosphorylation of various kinases activates NOX and increases ROS generation.

XANTHINE OXIDASE (XO)

XO is a major source of ROS. Xanthine oxidoreductase (XOR) degrades purines to generate uric acid. XOR is transcribed as xanthine dehydrogenase, which is converted to XO after oxidation of cysteine residues or proteolysis (Waud and Rajagopalan, 1976; Amaya et al., 1990; Kelley et al., 2010). XO generates both $O_2^{\bullet-}$ and H_2O_2, the latter in greater amounts under low O_2 and pH, e.g., inflammation, ischemia. Higher $O_2^{\bullet-}$ levels can be detected under low xanthine concentrations, and high O_2 tensions (Kelley et al., 2010). Landmesser et al. showed that Ang II administration elevates XO through ROS from NADPH oxidase. NADPH oxidase inhibition decreased XO and $O_2^{\bullet-}$ levels suggesting that Ang II stimulation of NOX proteins is needed for XO stimulation. Additionally, XO inhibitors like oxypurinol and tungsten markedly reduced Ang II-induced endothelial $O_2^{\bullet-}$, in agreement with the idea that Ang II stimulates XO. Furthermore, losartan (AT_1R blocker) treatment decreased the endothelial levels of both XO and $O_2^{\bullet-}$. Patients with coronary disease treated with AT_1R blocker for 4 weeks displayed reduced endothelial XO activation compared to the placebo (Landmesser et al., 2007).

OTHER ENZYMES

Other enzymes such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, glycerol-3-phosphate dehydrogenase, and from fatty acid β oxidation can also contribute to ROS generation (Kang and Pervaiz, 2012). Ang II can modulate the activity of these various enzymes. For instance, many studies have shown effects of Ang II on fatty acid oxidation and nonalcoholic fatty liver disease (Kurita et al., 2008; Toblli et al., 2008). Increased Ang II levels cause mitochondrial oxidative damage, which leads to impairment of beta oxidation causing hepatic steatosis. Treatment with AT_1R blockers like valsartan produced substantial improvement of mitochondrial abnormalities (Monteiro et al., 2005). The mechanism by which fatty acid oxidation appears to be mediated by ROS generated from NADPH oxidase as well as decreased Cu, Zn SOD activity (Wei et al., 2009). Ang II has also been shown to promote pyruvate dehydrogenase complex acetylation, leading to decreased glucose oxidation (Mori et al., 2013). α-ketoglutarate dehydrogenase activity was shown to be increased by Ang II treatment. Increased cytoplasmic and mitochondrial free Ca^{+2} levels are associated with hepatic stimulation via Ang II. Since α-ketoglutarate dehydrogenase function is positively stimulated by Ca^{+2}, its activity is indirectly influenced by Ang II (Exton, 1985; Williamson et al., 1985; Rashed et al., 1988).

Together, the evidence available indicates that RAS can induce ROS generation through direct—influencing various signaling pathways, as well as indirect—modulating activities of antioxidant enzymes, mechanisms.

AGING, MITOCHONDRIA AND RAS ASSOCIATED PATHOLOGY

Aging is associated with the accumulation of various deleterious changes in cells. According to the free radical and mitochondrial theory of aging, mitochondria initiate most of the deleterious changes in aging and govern life span (Harman, 1956, 2006; Cadenas and Davies, 2000; Cadenas, 2004). Three key mitochondrial functions that become dysregulated with aging are: (1) ROS production, (2) ATP synthesis, and (3) apoptosis (Conley et al., 2007). As proposed by the mitochondrial theory of aging, increased mitochondrial ROS generation precipitates mitochondrial DNA damage and mutations, which in turn leads to failed oxidative phosphorylation and diminished ADP/ATP reservoir (Harman, 1956, 2006), ultimately contributing to mitochondrial deterioration and activation of cell death pathways (Echtay et al., 2002; Dirks et al., 2006; Skulachev, 2006; Conley et al., 2007; Pandur et al., 2014). Moreover, mitochondrial morphodynamics through fusion and fission can also be altered with aging could potentially lead to their dysfunction (Bleazard et al., 1999; Chen et al., 2003; Yu et al., 2012). Chronically activated ROS has been implicated in mitochondrial energetic impairment along with the development and progression of a host of aging-related conditions including atherosclerosis, myocardial hypertrophy, vascular dysfunction, hypertension (Heymes et al., 1998; Wang et al., 2003; Kimura et al., 2005; Doughan et al., 2008; Fukai, 2009; Min et al., 2009; Widder et al., 2009), type 2 diabetes, frailty, heart failure, neurodegeneration (including Alzheimer's disease, Horan et al., 2012), and sarcopenia (Ballinger et al., 1994; Wallace, 2001, 2005, 2010, 2011; Loeb et al., 2005; Conley et al., 2007; Moore et al., 2010). Clinically, the onset of this mitochondrial failure is difficult

to estimate, however, the accumulation of damaged mitochondria typically appears in humans by mid to late seventies and once established is thought to be irreversible (Aiken et al., 2002; Herbst et al., 2007). Recently, several studies have shed light on early mitochondrial changes in healthy subjects that predate the accumulation of damaged mitochondria by almost a decade. These studies have demonstrated that increased uncoupling leads to a reduction in mitochondrial efficiency in otherwise healthy people in their sixties (Greco et al., 2003; Hutter et al., 2004; Bua et al., 2006; Mogensen et al., 2006; Amara et al., 2007).

The major mechanism by which dysfunction occurs is via mtDNA mutations. Unlike nuclear genome, mitochondrial genome is circular, and is not condensed around histones or packed tightly. This makes it less protected and more easily damageable than nuclear DNA (nDNA) (Croteau et al., 1999). mtDNA damage is mostly attributed to ROS. AngII can stimulate mitochondrial ROS production via activation of cytoplasmic NOX-derived $O_2^{\cdot-}$ and through direct effects on mitochondria as well. Administration of antioxidants inhibited Ang II effects on AP-1 signaling pathway (Puri et al., 1995; Xia et al., 1998; de Cavanagh et al., 2007). Further evidence in support of Ang II action on mitochondria is given by research showing AT_2R co-localization with this organelle likely in inner membrane, in various tissues. Ang II and AT_2R are likely generally present in the inner membrane of mitochondria (Inagami, 2011). Ang II stimulates production of $NO^{\cdot}$ via activation of calcium/calmodulin-dependent eNOS, including mRNA and protein expression levels (Brown, 1999; Yan et al., 2003). mtDNA codes for critical proteins participating in oxidative phosphorylation (Croteau et al., 1999). In addition, mtDNA has less repair mechanisms compared to nDNA; for example, mitochondria lack nucleotide excision repair mechanisms (Larsen et al., 2005). mtDNA mutation rate is shown to increase with age, affecting liver, skeletal muscles, and cardiac muscles (Katayama et al., 1991; Corral-Debrinski et al., 1992; Marin-Garcia et al., 2002; Druzhyna et al., 2008). Excess ROS generation may overwhelm antioxidant enzymes, thereby preventing the mitochondria from protecting themselves (Golden and Melov, 2001). With chronic exposure to elevated ROS and decline in repair mechanisms, mtDNA mutations accumulate during aging (Lin and Beal, 2006). Under RAS over-activation- as seen in diabetes, hypertension and aging- Ang II induced ROS plays a significant role in tissue damage. Ang II induced mtROS generation has been implicated in atherosclerotic lesions and impairment of cardiac respiration and TCA cycle function leading to disease (Pueyo et al., 2000; de Cavanagh et al., 2007).

In cardiovascular tissue, mitochondrial ROS contribute to senescence of endothelial cells and chronic low-grade vascular inflammation (Ungvari et al., 2007). These endothelial cells can contribute to atherosclerosis by suppressing regeneration and angiogenesis of endothelium in the vascular wall leading to cardiovascular aging (Dai et al., 2012) and the development of hypertension (Heymes et al., 2003; Shiomi et al., 2004; Wang et al., 2010; Sugamura and Keaney, 2011; Dikalov and Ungvari, 2013).

Development and progression of several neurodegenerative disorders has also been linked to mitochondrial dysfunction (Beal, 2007). A significant decrease in mitochondrial coupling efficiency in primary hippocampal neurons, reduced steady state basal respiration, and decreased ATP turnover were noted in several neurodegenerative disorders (Horan et al., 2012). In Alzheimer's disease, several studies have demonstrated that oxidative damage appears to increase development of intracellular Aβ plaques. ROS activation of c-Jun N terminal kinase and p38 mitogen activated protein kinase lead to increased activity of β secretase, causing increased Aβ levels (Nishida et al., 2006; Beal, 2007). Parkinson's disease is caused by a recessive mutation of DJ1 that leads to hypersensitivity to MPTP and oxidative stress since DJ1 protects against oxidative stress-induced cell death. Studies show that treatment with SOD1 and vitamin E decrease degeneration of dopaminergic neurons (Bonifati et al., 2003; Kim et al., 2005; Wang et al., 2006a). Huntington's disease (HD) is also associated with oxidative stress since reduced activity of Complex II and III is seen in basal ganglia and cortex. In addition, HD patients show decreased PGC1α, which suppresses ROS by activating ROS scavenging enzymes (St-Pierre et al., 2006; Weydt et al., 2006; Beal, 2007). ROS are implicated not only in neurodegenerative disorders but also in various other diseases like age-related musculoskeletal disorders. In sarcopenia, a disease characterized by reduced skeletal muscle mass with aging, electron transport system abnormalities were seen in muscle fibers with "ragged red" phenotype and these changes were associated with loss of muscle mass (Bua et al., 2002). Patients with Type 2 diabetes show decreased antioxidant capacity and possibly an increase in ROS generation by leukocytes (Mohanty et al., 2000). Studies show increased expression of ROS markers in pancreatic islet cells under diabetic conditions. Furthermore, β cells are much more sensitive to ROS due to decreased antioxidant capacity. Chronic hyperglycemia increases ROS levels while decreasing binding of the transcription factors PDX-1 and Maf-A from pancreas that exacerbates suppression of insulin synthesis and release. In addition, treatment with antioxidants such as N-acetyl-L-cysteine and taurine, diminished insulin resistance due to hyperglycemia (Kaneto et al., 2010).

The overlap between age related changes in RAS and mitochondria and the implications of this overlap on age-related diseases are quite complex. Studies shows that Ang II contributes to plaque rupture by initiation of VSMC apoptosis, which could be prevented by AT_1R blockers (Lemay et al., 2000). Ang II induces metalloproteinase activity, which is involved in collagen breakdown and matrix degradation, indirectly through ROS (Shah and Galis, 2001; Nickenig and Harrison, 2002). Ang II can induce interleukin-6 (IL6), leukemia inhibitory factor, and cardiotrophin-1 in cardiac fibroblasts (Sano et al., 2001). IL-6 is an inflammatory marker and a high level of which is associated with mortality due to increased progression of cardiovascular disease (Volpato et al., 2001). Leukemia inhibitory factor is a key player in cardiac hypertrophy (Kodama et al., 1997). Cardiotrophin-1 plays a role in heart failure since it can induce ventricular remodeling by activating cardiomyocyte hypertrophy and collagen synthesis (Calabro et al., 2009).

Many studies highlighted the role that RAS plays in hypertension and cardiovascular disease (Marchesi et al., 2008). By activating local mediators, like vascular endothelial growth factor (VEGF) and prostaglandins, such as leukotriene C4, PGE2, and

PGI2, Ang II plays a critical role in regulating vascular permeability during hypertension (Harris et al., 2004). Ang II-stimulated release of these local factors in VSMCs leads to angiogenesis, vascular permeability, and inflammation. In particular, studies have shown that AT_1R activation causes VEGF secretion (Suzuki et al., 2003). Hypertension is a condition that results from ROS of vascular origin produced by elevated levels of Ang II. Chronic administration of Ang II in mice, triggered elevated $O_2^{\cdot-}$ from mitochondria as compared to controls (Widder et al., 2009). $O_2^{\cdot-}$ and H_2O_2 produced due to elevated Ang II can carry out various actions in VSMCs—phosphorylation of MAP kinases, induction of proto-oncogenes, and activation of AP-1; H_2O_2 can induce PDGF stimulation of STATS. All these signaling events contribute to vascular wall remodeling and thickening seen in hypertension (Touyz, 2000). Ang II via AT_1R also affects brain tissue by increasing neuronal firing rate and activity. In neurons, NADPH oxidase-derived ROS produced due to Ang II signaling increase intracellular Ca^{+2} concentration (Wang et al., 2006b), which in turn can stimulate mitochondrial $O_2^{\cdot-}$ stimulates mitochondrial superoxide generation (Hongpaisan et al., 2004). In rostral ventrolateral medulla, these effects may influence blood pressure, causing baroreflex abnormalities in chronic hypertension (Nozoe et al., 2008).

RAS has been implicated in many pathological conditions. Ang II can precipitate mild to severe mitochondrial dysfunction in addition to ROS generation. Amelioration of age-related renal mitochondrial dysfunction under hypertensive conditions, and ischemic injury, has been described in patients treated with angiotensin receptor blockers (Doughan et al., 2008). Losartan treatment prevents mitochondrial dysfunction and structural changes in the kidney while up regulating antioxidant enzymes, maintaining GSH and MnSOD levels, and attenuating uncoupling proteins. These effects were not apparent when patients were treated with a Ca^{2+} channel blocker allowing to conclude that Ang II must play a role in mitochondrial dysfunction (de Cavanagh et al., 2006).

Since Ang II is linked to NOX, Wosniak et al. (2009) examined the effects of mild mitochondrial uncoupling and Ang II stimulation of NOX isoform in VSMCs. Ethidium bromide was used to induce mild mitochondrial stress, which the investigators described as "neither rapidly lethal nor promoting profound redox derangements" (Wosniak et al., 2009). Results showed that Ang II-induced NOX activation can be completely eliminated with mild mitochondrial uncoupling. Ang II up regulates NOX 1 expression and down regulates NOX4 expression and with mild mitochondrial dysfunction there was a decrease in NOX1 and increase in NOX4. Therefore, these authors concluded that functional mitochondria are required for Ang II-induced NOX activation. Wosniak et al. also noted that mitochondrial function influences activation of growth factor receptors involved in NOX signaling (Wosniak et al., 2009). In addition, when Ang II was administered, increased mtDNA damage was only observed in those cells that had existing dysfunctional mitochondria. Cells with functional mitochondria did not show any markers of mtDNA damage. The researchers speculated that mitochondria might be acting as a switch between normal to pathological effects of Ang II (Wosniak et al., 2009). The relationship between angiotensin-related mitochondrial ROS and NADPH oxidase is still a novel area of research that can potentially provide insight into pathophysiology of many diseases and may pave the way for new therapeutic approaches.

THE USE OF ANGIOTENSIN RECEPTOR BLOCKERS (ARBs) IN MITOCHONDRIAL DYSFUNCTION

Elevated Ang II produces increased levels of ROS (by activating NADPH oxidase and various other enzymes) that contribute to various pathological conditions. AT_1R blockade decreases RAS-mediated activation of NADPH oxidase and oxidative stress, leading to reduced left ventricular fibrosis and mitochondrial remodeling (Whaley-Connell et al., 2008). Losartan treatment also reverses left ventricular hypertrophy, reduces fibrosis, ultimately causing an overall improvement of cardiac function (Khaper and Singal, 2001). Various studies have shown a lower rate of mortality from cardiovascular disease in patients treated with angiotensin receptor antagonists. Beneficial effects of angiotensin receptor blockers include lowered cerebral lesion incidence, reduced cardiac hypertrophy, and reduced glomerulosclerosis. Eprosartan was shown to be effective in preventing cardiac remodeling, renal failure, and decreasing mortality (Takemori et al., 2005). Interestingly, insulin treatment induces AT_1R overexpression, and in diabetics, ARBs have been shown to effectively preserve renal function and reduce cardiovascular endpoints (Nickenig et al., 1998; Nickenig, 2004). ARBs reduces Ang-II induced lipolysis and adipocyte dysfunction (Takemori et al., 2013). Contrary to AT_1R, the effects of AT_2R are considered protective. ARBs may increase activation of AT_2 receptor augmenting end-organ protection (Carey et al., 2000; Unger, 2002). More research on ARBs and activation of AT_2 receptor might yield promising results in treating various pathological conditions.

AngII receptor blockers have several features in common: high affinity for AT_1R and almost no affinity for AT2 receptors; high protein binding capacity behaving as competitive inhibitors with slow dissociation (Burnier, 2001). There are six common types of ARBs used currently as treatment for hypertension—losartan, valsartan, candesartan cilexetil, irbesartan, eprosartan, and telmisartan. These ARBs have been shown to protect against various disease states. For example, in rats with myocardial infarction, losartan improves function of several antioxidant enzymes, significantly reducing oxidative stress. In particular, losartan increases the activity of GSH peroxidase (Khaper and Singal, 2001) while decreasing levels of vascular $O_2^{\cdot-}$ due to its antagonist activity on AT_1 receptors (Kurz et al., 1999). It also restores $NO^{\cdot}$ synthesis and availability playing its role as an antihypertensive drug protecting endothelial cells (Qadri et al., 2001). Valsartan is effective in improving heart mitochondrial function under acute ischemia (Monteiro et al., 2005). Long-term treatment of rabbit heart with valsartan, after infarction, showed reduced lipid peroxide levels; an improvement in postinfarct ventricular remodeling and coronary endothelial dysfunction was also seen (Kuno et al., 2002). In diabetic patients, candesartan cilexetil has been shown to improve pancreatic β cell function (Qadri et al., 2001) and irbesartan was effective against postprandial hyperglycemia and hypertriglyceridemia on endothelial function (Ceriello et al., 2005). Telmisartan can inhibit apoptosis, oxidative stress, and

neuro-inflammation in addition to its antihypertensive functions (Beckman et al., 1990; Butler et al., 2003; Monteiro et al., 2005; Shao et al., 2006). In hypertensive patients, ARBs were shown to improve normal retinal perfusion and endothelium-dependent vasodilation in coronary and renal circulation (Delles et al., 2004). In addition, ARBs have been associated with a reduction in inflammatory markers. This is of particular importance since low grade chronic inflammation is associated with many neurodegenerative diseases like Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis, multiple sclerosis, Huntington's disease and frailty (Tracy, 2003; Gao and Hong, 2008; Holmes et al., 2009). ARBs are also able to reverse early myocardial impairment (Cadeddu et al., 2010), improve insulin sensitivity, decrease incidence of type 2 diabetes (Saitoh et al., 2009) and prevent renal fibrosis (Shao et al., 2006).

PROSPECTIVE

Mitochondrial dysfunction and oxidative stress underlie many pathologies and constitute primary theories of aging. Understanding how age-related mitochondrial dysfunction might be mitigated or exacerbated is critical to advancing this research field. The RAS is currently regarded as a physiological system of vital importance because of its links to both mitochondrial function/dysfunction and a host of age-related diseases. Although many prior studies have advanced our understanding in each of these aging-relevant biological systems, the progress in delineating the molecular mechanisms involved has been rather slow. Given the availability of selective, and relatively safe blockers of RAS, studies focusing on the interface between mitochondria, RAS, and aging may prove to be very important in clinical translation of research.

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Hypertrophic cardiomyopathy: a heart in need of an energy bar?

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Hypertrophic cardiomyopathy (HCM) has been recently recognized as the most common inherited cardiovascular disorder, affecting 1 in 500 adults worldwide. HCM is characterized by myocyte hypertrophy resulting in thickening of the ventricular wall, myocyte disarray, interstitial and/or replacement fibrosis, decreased ventricular cavity volume and diastolic dysfunction. HCM is also the most common cause of sudden death in the young. A large proportion of patients diagnosed with HCM have mutations in sarcomeric proteins. However, it is unclear how these mutations lead to the cardiac phenotype, which is variable even in patients carrying the same causal mutation. Abnormalities in calcium cycling, oxidative stress, mitochondrial dysfunction and energetic deficiency have been described constituting the basis of therapies in experimental models of HCM and HCM patients. This review focuses on evidence supporting the role of cellular metabolism and mitochondria in HCM.

Keywords: hypertrophic cardiomyopathy, mitochondria, calcium handling, bioenergetic deficit, induced pluripotent stem cells (iPSCs)

CLINICAL FEATURES OF HYPERTROPHIC CARDIOMYOPATHY

Hypertrophic cardiomyopathy (HCM) was first recognized as a clinical entity, approximately 55 years ago (Brock, 1957; Teare, 1958; Cohen et al., 1964; Ross et al., 1966). It is the most common inherited cardiac disease with an estimated prevalence of 1: 500 in young individuals (Maron, 2002). Inheritance is autosomal dominant, with variable penetrance in 50–60% of patients; causal mutations have not been identified in 40–50% of HCM patients (Jarcho et al., 1989; Solomon et al., 1990; Marian and Roberts, 2001; Marian, 2002). Nine different chromosomal loci have been linked to HCM with the majority of genes encoding cardiac sarcomeric proteins (Jarcho et al., 1989; Geisterfer-Lowrance et al., 1990; Watkins et al., 1993; Thierfelder et al., 1994). The most common mutations occur in genes encoding for β-myosin heavy chain (35%), myosin binding protein C (20%), troponin T (5%) and α-tropomyosin (<3%), which have roles in cardiac excitation-contraction coupling (Maron and Maron, 2013).

Asymmetric ventricular hypertrophy and left ventricular outflow tract obstruction with normal or hyperdynamic systolic function are common morphologic manifestations of HCM (Maron et al., 2003). However, clinical phenotype is variable even among individuals carrying the same causal mutation due to effects of modifier genes, which are largely unknown (Seidman and Seidman, 2001). As a result degree and location (mid-ventricular, septal, apical and concentric) of hypertrophy and obstruction are variable in patients with HCM (**Figure 1**). Clinical presentation is also heterogeneous, spanning the spectrum from individuals who are largely asymptomatic, to patients with moderate to severe symptoms, ranging from angina, exercise intolerance to heart failure (requiring heart transplantation), atrial fibrillation and sudden cardiac death (Maron, 2002; Maron et al., 2002; Gersh et al., 2011). Irrespective of the causal mutation, pathologically, HCM is characterized by myocyte hypertrophy, myocyte disarray and fibrosis (Ho et al., 2010).

Pioneering work by several groups has revealed the molecular genetics and biophysical mechanisms underlying HCM. A variety of functional defects, including altered Ca^{2+} sensitivity and/or affinity, myofibrillar ATPase activity, Ca^{2+} handling, cross-bridge dynamics, impaired energetics, oxidative stress and electrophysiologic abnormalities have been identified in experimental models (Straceski et al., 1994; Spindler et al., 1998; Blanchard et al., 1999; Gao et al., 1999; Georgakopoulos et al., 1999; Tardiff et al., 1999; Solaro et al., 2002; Javadpour et al., 2003; Adhikari et al., 2004; Szczesna-Cordary et al., 2004; Ertz-Berger et al., 2005; Hernandez et al., 2005; Robinson et al., 2007; Greenberg et al., 2009, 2010; Guinto et al., 2009; Mettikolla et al., 2011; Puglisi et al., 2014) and patients (Haq et al., 2001; Crilley et al., 2003; Nakamura et al., 2005; Dimitrow et al., 2009; Unno et al., 2009; Ho et al., 2010; Bravo et al., 2012; Coppini et al., 2013; Lin et al., 2013; Gruner et al., 2014). Since HCM-causing mutations increase the energetic cost of tension development, it has been hypothesized that excessive sarcomeric energy use leads to the HCM phenotype (Blair et al., 2001; Crilley et al., 2003; Abozguia et al., 2010). We (Abraham et al., 2013) and others (Jung et al., 1998, 2000; Crilley et al., 2003; Timmer et al., 2011) have shown reduced PCr/ATP ratios in HCM patients with both established left ventricular hypertrophy and in the pre-hypertrophic stage, which suggests that bioenergetic deficits may be a primary cause of myocardial remodeling.

EVIDENCE OF HCM AS A METABOLIC DISEASE

^{31}P NMR spectroscopy studies have demonstrated a reduction in ATP reserve in HCM mouse models following inotropic

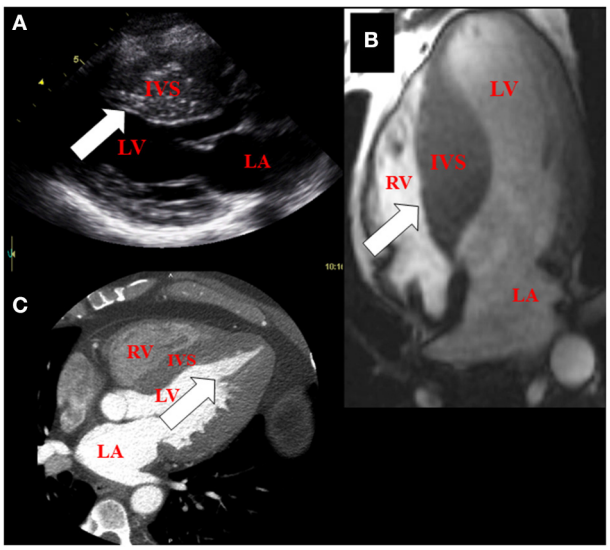

FIGURE 1 | Imaging features of hypertrophic cardiomyopathy using multi-modality cardiac imaging in patients. (A) Basal hypertrophy of the inter-ventricular septum (arrow) using echocardiography (parasternal long axis view of the heart). **(B)** Mid-septal hypertrophy (arrow) using magnetic resonance imaging (4-chamber view of the heart). **(C)** Apical hypertrophy (arrow) using Computed tomography (4-chamber view of the heart). LA, left atrium; IVS, inter-ventricular septum; LV, left ventricle; RV, right ventricle.

stimulation (Spindler et al., 1998; Javadpour et al., 2003). Evidence for energy deficit in HCM has also been obtained from patient studies revealing increased glucose uptake (Tadamura et al., 1996), reduction of PCr/ATP ratios in pre-hypertrophic patients (Crilley et al., 2003) and reduced coronary sinus pH despite non-limiting capillary oxygen pressures (possibly indicating up-regulation of glycolysis with lactate generation) (Tadamura et al., 1996; Jung et al., 1998; Ashrafian et al., 2003; Keren et al., 2008). However, it is not known whether the energy deficit paradigm can be generalized to *all* HCM patients, at *all* stages of the disease. Furthermore, the molecular basis of the energetic deficits in HCM and their attendant consequences has been understudied.

In the heart, ATP supply is tightly regulated to meet energetic demands of the myofilaments. The mechanisms by which cardiac energetics is finely tuned are still a matter of considerable debate, but there is emerging consensus on the importance of two regulators, Ca^{2+} and ADP (Cortassa et al., 2006; Saks et al., 2006; Balaban, 2009). During contraction, Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum floods the cytoplasm where it binds the thin filament regulatory protein Troponin C, thereby initiating contraction (Bers, 2002). Coordinate activation of ATP production arises because mitochondria, positioned close to the SR, take up Ca^{2+} via the mitochondrial calcium uniporter (MCU) (Maack and O'Rourke, 2007). Mitochondrial matrix calcium regulates 3 key enzymes in the tricarboxylic acid (TCA) cycle that harnesses the decarboxylation of acetyl-CoA to yield reduced nicotinamide adenine dinucleotide (NADH) which fuels the respiratory electron transport chain (ETC) and is converted to NADPH which plays a critical role in maintaining mitochondrial anti-oxidant capacity (McCormack and Denton, 1990; Hansford and Zorov, 1998; Liu et al., 2014); Mitochondrial Ca^{2+} can also directly stimulate respiratory complex activity, including the mitochondrial ATP synthase (*F1F0* ATPase) (Territo et al., 2000). Thus, Ca^{2+} coordinately regulates ATP-consuming myofilaments and ATP-generating oxidative phosphorylation (**Figure 2**).

Ca^{2+} uptake by mitochondria is dependent on cytosolic Na^{+} levels, which has been demonstrated to be elevated in experimental models of heart failure (Liu and O'Rourke, 2008) and failing human hearts (Pieske and Houser, 2003). The O'Rourke group has demonstrated that elevated cytosolic Na^{+} increases the rate of the mitochondrial Na^{+}—Ca^{2+} exchanger (mNCE), which promotes mitochondrial Ca^{2+} efflux and decreases the mitochondria's ability to accumulate Ca^{2+} during conditions of high demand (Maack et al., 2006; Liu and O'Rourke, 2013). Without Ca^{2+}-induced Kreb's cycle stimulation, NADH and NADPH become more oxidized and are unable to recharge antioxidant systems, leading to ROS accumulation in the mitochondrial matrix and release into the cytosol (Kohlhaas et al., 2010; Gauthier et al., 2013; Liu and O'Rourke, 2013). Partial inhibition of mNCE by CGP-37157 attenuated adverse ventricular remodeling and was anti-arrhythmic in a guinea pig model of pressure overload (Liu et al., 2014). A recent study of Ranolazine, an inhibitor of late Na^{+} current, that is known to contribute to cytosolic Na^{+} overload revealed salutary effects on action potential duration and arrhythmias in cardiac myocytes of HCM patients who underwent myectomy (Coppini et al., 2013). Investigation of cytosolic Na^{+} levels (Gao et al., 2013) and mitochondrial Ca^{2+} handling is needed in order to assess whether altered mitochondrial Ca^{2+} dynamics contribute to energetic deficits and oxidative stress in HCM (Nakamura et al., 2005; Senthil et al., 2005; Marian et al., 2006; Dimitrow et al., 2009).

Conditions such as exercise that quickly elevate heart rate impose energetic demands that can quickly exceed Ca^{2+}-regulated supply. In these cases, the by-product of myofilament ATPase activity, ADP, provides vital feedback stimulation of energy in two ways. Firstly the original work by Britton Chance and colleagues showed that the rate of ATP formation by Complex V is driven by the concentration of ADP. Myofilaments also possess a local ATP-buffering capacity maintained by cytosolic creatine kinase (CK). In high work conditions, ADP accumulation is sensed by CK, which catalyzes phosphoryl group transfer from phosphocreatine to regenerate ATP (Saks et al., 2006; Balaban, 2009) (**Figure 2**). Our studies in patients from a family carrying the R403Q mutation in myosin heavy chain (MHC) revealed a 43% reduction in forward CK flux at rest, indicating reduced metabolic reserve (Abraham et al., 2013). A recent study by Critoph et al. revealed reduced cardiac reserve secondary to blunted increase in cardiac output, in HCM patients undergoing exercise stress testing (Critoph et al., 2014). NMR studies in mice with R403Q-MHC (Spindler et al., 1998) and R92-TNT (Javadpour et al., 2003; He et al., 2007) mutations verified impaired myocardial energetics during inotropic stimulation. The decreased [PCr], increased [Pi], [ADP] and unchanged or decreased [ATP] can result in reduction in the calculated free energy release from ATP hydrolysis ($1\Delta G$) (Spindler et al., 1998)

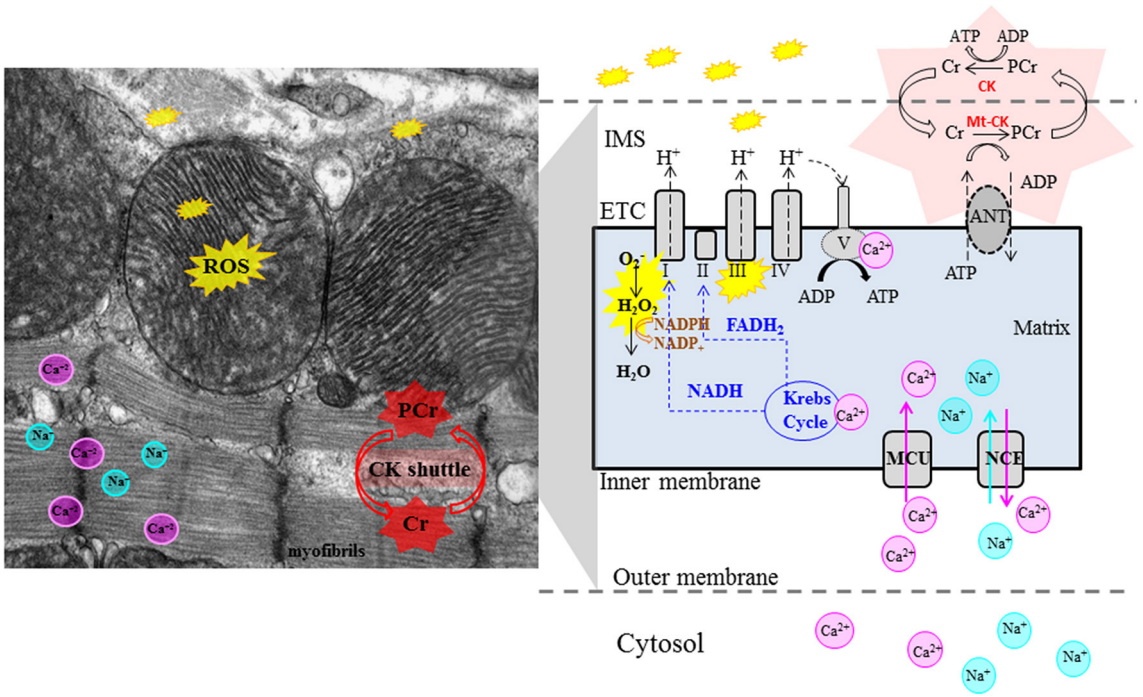

FIGURE 2 | Role of Mitochondria in pathogenesis of cardiac phenotype in HCM. Left panel: electron microscopy image of mouse heart. Right panel: schematic illustrating mitochondrial physiology. The Krebs cycle generates reducing equivalents (NADH, $FADH_2$) that drive proton pumping, establish the proton-motive force across the mitochondrial inner membrane and contribute to ROS scavenging. Mitochondrial ATP synthase (complex V) couples proton influx to ATP generation. Matrix concentrations of Ca^{2+} and Na^+ play an important role in control of oxidative phosphorylation. Mitochondria are the main source of ATP generation and important source of ROS (from complexes I and III) in cardiac myocytes. Abnormalities in mitochondrial function, reduced CK flux, oxidative stress and impaired Ca^{2+} handling have been implicated in generation of the cardiac phenotype in HCM. Cr, creatine; PCr, creatine-phosphate, Mt-CK, mitochondrial creatine kinase; ANT, Adenine nucleotide translocator; ETC, electron transport chain; IMS, inter-membrane space; MCU, mitochondrial calcium uniporter; NCE, mitochondrial Na^+–Ca^{2+} exchanger.

which in turn can impair the function of cellular ATPases (e.g., myosin ATPase, Na^+–K^+-ATPase) and Ca^{2+} pumps like SERCA, leading to systolic and/or diastolic dysfunction, reduction or blunted increase in stroke volume, increased levels of cytosolic Na^+, Ca^{2+} and arrhythmias under conditions of high work load, such as intense exercise (Unno et al., 2009; Ashrafian et al., 2011; Watkins et al., 2011).

THE ROLE OF MITOCHONDRIA IN HCM: WHAT WE KNOW

The vital role of mitochondria as providers of energy for the high demands of cardiac contractility is well recognized, as is their contribution to necrotic and apoptotic cell death (Seddon et al., 2007). More, recently, the novel role of mitochondria as signaling organelles has emerged, primarily through their ability to produce reactive oxygen species (ROS) -including superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH)- and reactive nitrogen species, including nitric oxide (NO) and peroxynitrite ($ONOO^-$) (Balaban et al., 2005; Figueira et al., 2013). ROS are best known for the damage they cause by directly oxidizing proteins, lipids, and DNA, but recent evidence suggests that the controlled and carefully modulated release of ROS from the mitochondrial network can activate specific signaling pathways or mediate reversible post-translational modifications of target proteins with pronounced effects on function (Terentyev et al., 2008; Bayeva and Ardehali, 2010). Additionally, because mitochondria are major determinants of the redox potential of both the pyrimidine nucleotide (NADH, NADPH) and thiol (GSH, thioredoxin) pools, they are important regulators of myocyte function (Stanley et al., 2011; Kembro et al., 2013; Liu and O'Rourke, 2013; Liu et al., 2014). However, very little is known about myocyte and mitochondrial redox potential in HCM (**Figure 2**).

Impairment of mitochondrial function and morphological disorganization has been reported in mouse models (Tardiff et al., 1999; Lucas et al., 2003) and in HCM patients (Unno et al., 2009). However, a systematic study of mitochondrial function is lacking. It is also unclear whether mitochondrial abnormalities are a primary event or secondary event in HCM. Of note, patients with mutations in mitochondrial DNA (Obayashi et al., 1992; Rotig et al., 1997; Okajima et al., 1998; Elliott and McKenna, 2004) can have a similar cardiac phenotype as HCM patients with sarcomeric protein mutations, suggesting that energetic deficits can lead to the cardiac phenotype of HCM.

The normal heart relies primarily on fatty acid oxidation for ATP generation (Abozguia et al., 2006; Ingwall, 2009). Pathologic hypertrophy is known to be associated with a reduction in fatty acid oxidation and increased reliance on glucose for ATP generation (Abozguia et al., 2006; Coppini et al., 2013). Positron emission tomography (PET) using ^{11}C-acetate and 18FDG have

been employed to study glucose and fatty acid oxidation in HCM patients (Grover-McKay et al., 1989; Nienaber et al., 1993; Perrone-Filardi et al., 1993; Tadamura et al., 1996; Tuunanen et al., 2007): some studies have found decrease/no change or increase in glucose uptake and the same is true for fatty acid oxidation, in hypertrophied and non-hypertrophied walls, when compared to controls. This may be attributable to differences in clinical characteristics (stage of disease, degree of hypertrophy, presence of microvascular dysfunction) and mutation status of the small number of patients who were studied. Since enzymes for fatty acid oxidation are located in mitochondria, it is unclear whether abnormalities in fatty acid oxidation are secondary to mitochondrial dysfunction. Metabolomic studies (Mayr, 2008) are needed in mouse models and HCM patients to obtain insights into metabolic remodeling and its role in generation of the cardiac phenotype in HCM.

ARRHYTHMIAS IN HCM—DO MITOCHONDRIA PLAY A ROLE?

Sudden cardiac death is the most dreaded and tragic phenotype, as it is often the first manifestation of the disease and occurs in asymptomatic and apparently healthy young individuals (Maron and Maron, 2013). The enhanced ventricular arrhythmogenicity has been attributed to abnormal cardiomyocyte orientation and alignment (disarray), microvascular ischemia, and fibrosis (Coppini et al., 2013).

Sarcomeres are known to sequester Ca^{2+} (bound: free ratio is 100:1). It has been hypothesized that HCM mutations may increase "Ca^{2+} trapping," and through altered on–off kinetics may lead to altered Ca^{2+} signaling and arrhythmogenesis (Semsarian et al., 2002; Ashrafian et al., 2011).

Another possibility that has not been explored is energetic deficits, because energy compromise would be most marked when the heart is subject to increased work load, as is the case during high intensity exercise. In fact, e*xercise-induced* arrhythmias are common causes of sudden death and defibrillator discharges in HCM patients (Ommen and Gersh, 2009; Spirito et al., 2014). Since HCM is associated with high sarcomeric ATP consumption at rest, the ability of the heart to provide sufficient ATP for myosin ATPase, SERCA and membrane ATPases could be compromised during exercise, leading to cytosolic Na^+ and Ca^{2+} overload and triggered activity leading to clinical arrhythmias (Watkins et al., 2011). Another possibility is the "*metabolic sink hypothesis*," proposed by the O'Rourke group (Akar et al., 2005), wherein regional oxidative stress (ROS-induced ROS release) in mitochondria results in mitochondrial membrane depolarization, K_{ATP}– channel opening and *reentrant arrhythmias* (Zorov et al., 2000, 2006; Aon et al., 2003, 2006; O'Rourke et al., 2005; Zhou et al., 2009; Cortassa et al., 2014).

THERAPIES IN HCM—ALL LEFT VENTRICULAR HYPERTROPHY IS NOT CREATED EQUAL

There is a need for therapies that prevent/reverse the cardiac phenotype in HCM (Force et al., 2010). Drugs such as beta-adrenergic antagonists (e.g., Metoprolol), L-type Ca^{2+} channel blockers (e.g., Diltiazem, Verapamil) (Semsarian et al., 2002; Elliott and McKenna, 2004; Spirito and Autore, 2006), angiotensin II receptor antagonists (e.g., Losartan), (Lim et al., 2001; Lombardi et al., 2009; Shimada et al., 2013) carnitine palmitoyltransferase-1/2 inhibitor (Perhexiline) (Abozguia et al., 2010), antiarrhythmics (e.g., Disopyramide, Amiodarone), surgical myectomy and alcohol septal ablation (Sorajja et al., 2012) have been used to treat symptomatic HCM (Gersh et al., 2011). Antioxidant therapy with L-NAC was shown to prevent hypertrophy and fibrosis in experimental models of HCM (Marian et al., 2006) and is now in clinical trials (HALT-HCM study). Recently, there has been interest in the use of Ranolazine, based on beneficial effects on action potential duration and arrhythmias, in cardiac myocytes derived from HCM patients undergoing myectomy that exhibited evidence of electrophysiologic remodeling (increased late Na^+ and Ca^{2+} currents, reduced repolarizing K^+ currents) (Coppini et al., 2013). Two studies are currently under way to test the efficacy of ranolazine on exercise tolerance and diastolic function in symptomatic HCM patients (RESTYLE-HCM, Germany, Menarini) and to treat chest pain or dyspnea in patients with HCM (RHYME, USA) (Spoladore et al., 2012). However, it is not known whether cytosolic Na^+ is increased early in the course of the disease (pre-hypertrophic stage) and whether it leads to mitochondrial dysfunction in any/all HCM mutations, or whether high levels of cytosolic Na^+ occur after the onset of myocyte hypertrophy and/or symptoms. Most importantly, *none of the agents tested clinically have been demonstrated to change disease course in symptomatic patients* (Nagueh et al., 2010). Possible reasons may be that the pathophysiology of myocyte hypertrophy is mutation-specific and the extent of hypertrophy (a common clinical endpoint) is only one determinant of prognosis. Another possibility is that all HCM is not created equal and consequently, *individualized, mutation-specific therapies* need to be developed.

Hypertrophy is a compensatory response to myocardial injury. While hypertension and HCM can both cause left ventricular hypertrophy which may be indistinguishable by clinical imaging, the molecular mechanisms underlying myocyte hypertrophy are probably different based on an early study of cyclosporine, which prevented left ventricular hypertrophy in the TAC (transverse aortic constriction) model (that simulates increased afterload caused by hypertension) (Sussman et al., 1998), but expedited hypertrophy in HCM mice with a mutation (R403Q) in the α-MHC gene (Teekakirikul et al., 2010). Hence there is *need for further investigations to clarify the mechanisms underlying the cardiac phenotype in HCM in order to spur development of new therapeutic strategies and pre-clinical screening tests*.

FUTURE DIRECTIONS

Identification of mutations has defined the genetic causes of HCM in 50–60% of HCM patients, but the molecular mechanisms underlying myocyte hypertrophy, fibrosis and ventricular arrhythmias have not been completely elucidated (Force et al., 2010). It is unclear to what extent genetic variants of HCM exhibit a common mechanism of pathogenesis and to what extent they differ. It is also unknown why certain sarcomeric mutations are well tolerated while others are particularly pernicious in patients, but not in animal models.

Based on positive results in animal models, clinical trials have investigated Ca^{2+} channel blockers and inhibitors of the

renin-angiotensin-aldosterone system in the HCM population, with limited success -possible reasons include differences in disease pathophysiology between HCM-causing mutations and differences in physiology between mouse and human myocytes. Hence, studies in human myocytes are needed to confirm results obtained in mouse models and develop therapies that modify the clinical course of disease. Since human heart tissue can only be obtained by heart biopsy or during surgery, it has been difficult to conduct human studies of disease pathophysiology in large numbers of HCM patients at various stages of disease.

Advances in IPSC (induced pluripotent stem cell) technology permit derivation of *human* cardiac myocytes obtained by differentiation of human IPSCs derived from HCM patients (Matsa et al., 2014). A recent study by the Wu group at Stanford demonstrated that myocytes differentiated from IPSCs (IPSC-CMs) recapitulate the HCM disease phenotype and can serve as a platform to test therapies (Lan et al., 2013). Furthermore, mitochondrial dysfunction resulting from low levels of Frataxin was also reproduced in IPSC-CMs derived from patients with Friedrich's ataxia (Hick et al., 2013). Hence, IPSC-CMs derived from HCM patients could serve as human model systems of HCM to investigate mitochondrial function and molecular mechanisms underlying cardiac phenotype, develop individualized screening tests and drug therapies in HCM patients with *known and unknown causal mutations*.

CONCLUDING REMARKS

HCM is caused by mutations in sarcomeric proteins in 50–60% of patients. These mutations have been shown to increase the energetic cost of tension development. However, it is unclear whether energetic deficits are involved in generation of the cardiac phenotype in *all* HCM patients and whether mitochondrial dysfunction precedes development of energetic deficits. Further investigation of mitochondrial function, metabolism and its relationship to cardiac function and electrophysiology in animal models of HCM and/or patient-derived myocytes is needed to clarify the molecular mechanisms underlying the cardiac phenotype in HCM and to design therapies that prevent, arrest and reverse the disease phenotype.

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A deficiency of apoptosis inducing factor (AIF) in Harlequin mouse heart mitochondria paradoxically reduces ROS generation during ischemia-reperfusion

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Background and Aims: AIF (apoptosis inducing factor) is a flavin and NADH containing protein located within mitochondria required for optimal function of the respiratory chain. AIF may function as an antioxidant within mitochondria, yet when released from mitochondria it activates caspase-independent cell death. The Harlequin (Hq) mouse has a markedly reduced content of AIF, providing an experimental model to query if the main role of AIF in the exacerbation of cell death is enhanced mitochondrial generation of reactive oxygen species (ROS) or the activation of cell death programs. We asked if the ROS generation is altered in Hq heart mitochondria at baseline or following ischemia-reperfusion (IR).

Methods: Buffer perfused mouse hearts underwent 30 min ischemia and 30 min reperfusion. Mitochondrial function including oxidative phosphorylation and H_2O_2 generation was measured. Immunoblotting was used to determine the contents of AIF and PAR [poly(ADP-ribose)] in cell fractions.

Results: There were no differences in the release of H_2O_2 between wild type (WT) and Hq heart mitochondria at baseline. IR increased H_2O_2 generation from WT but not from Hq mitochondria compared to corresponding time controls. The complex I activity was decreased in WT but not in Hq mice following IR. The relocation of AIF from mitochondria to nucleus was increased in WT but not in Hq mice. IR activated PARP-1 only in WT mice. Cell injury was decreased in the Hq mouse heart following *in vitro* IR.

Conclusion: A deficiency of AIF within mitochondria does not increase ROS production during IR, indicating that AIF functions less as an antioxidant within mitochondria. The decreased cardiac injury in Hq mouse heart accompanied by less AIF translocation to the nucleus suggests that AIF relocation, rather than the AIF content within mitochondria, contributes to cardiac injury during IR.

Keywords: reactive oxygen species, electron transport chain, apoptosis, poly(ADP-ribose)

INTRODUCTION

Apoptosis inducing factor (AIF) is a nuclear encoded protein synthesized as a 67 kDa precursor (Sevrioukova, 2011; Natarajan and Becker, 2012). The mature form of AIF (62 kDa) is located within the mitochondrial intermembrane space following import and removal of the mitochondrial localization signal (Sevrioukova, 2011; Natarajan and Becker, 2012). AIF has a pro-survival role when it is located within mitochondria, whereas release of AIF from mitochondria into cytosol followed by nuclear import activates caspase-independent cell death (Sevrioukova, 2011; Natarajan and Becker, 2012). The presence of FAD and NAD cofactors in the mature AIF renders it a potential antioxidant within the mitochondrial intermembrane space (Klein et al., 2002; van Empel et al., 2005), although this concept has been challenged (Sevrioukova, 2011). Nonetheless, the presence of these cofactors strongly suggests that AIF responses both within mitochondria and following release are likely responsive to and modulated by the local redox environment (Sevrioukova, 2011). The lower expression of AIF in Harlequin (Hq) mice impacts metabolism and response to tissue stress, in an organ-dependent manner (Klein et al., 2002; Vahsen et al., 2004). AIF deletion is embryonic lethal (Klein et al., 2002). When activated following birth, AIF deletion in heart and skeletal muscle leads to profound dilated cardiomyopathy and muscle wasting due to a marked decrease in electron transport complex I activity concomitant with increased reactive oxygen species (ROS) production from mitochondria (Joza et al., 2005; Pospisilik et al., 2007). Attenuation of the severity of AIF deficiency through extensive backcross breeding or the use of (female) heterozygotes leads to more modest defects in mitochondrial respiration that do not

exhibit increased ROS production in the baseline state, and in fact display potential resistance to exogenous disease (Pospisilik et al., 2007) suggestive of protective modulation of metabolism (Chen et al., 2007; Oxler et al., 2012). Thus, consistent with observations in other genetic models of partial complex I deficiency, a protective response to tissue injury may be observed (Oxler et al., 2012).

The Hq mouse exhibits an approximately 80% decrease in AIF content in all tissues. Defects are most profound in brain, with decreased complex I activity and complex I dependent respiration (Klein et al., 2002) and the development of disease in retina and brain (Hisatomi et al., 2001), reminiscent of human mitochondrial disease (Sevrioukova, 2011). In contrast, in heart, the metabolic defects are more subtle, although increased cardiac injury following *in vivo* ischemia-reperfusion (IR) and an increased susceptibility to heart failure in an aortic banding model of cardiac pressure overload have been reported (van Empel et al., 2005). The capacity to scavenge ROS is reported to be decreased in Hq mouse heart mitochondria compared to wild type (van Empel et al., 2005), suggesting that AIF has a potential antioxidant role. However, the net release of H_2O_2 is not altered in Hq mouse brain mitochondria compared to wild type (Chinta et al., 2009). This finding does not support an antioxidant role for AIF within mitochondria.

Cardiac mitochondria provide the energy to support heart function, whereas diseased and disabled mitochondria are a source of cardiomyocyte damage (Lesnefsky et al., 2001; Gustafsson and Gottlieb, 2008; Murphy and Steenbergen, 2008). IR results in damage to the electron transport chain that serves as a key source of ROS that exacerbate cardiac injury (Turrens, 2003; Chen et al., 2007). The net release of ROS from intact mitochondria represents a balance between ROS generation and the capacity of mitochondrial antioxidants (Turrens et al., 1991; Rigobello et al., 2006; Wenzel et al., 2008; Stanley et al., 2011). In the present study, we investigated if the net release of ROS from Hq mouse heart mitochondria is increased compared to wild type at baseline and if the genetic knockdown of AIF in Hq mice affects the net ROS generation and susceptibility to injury in the heart following the tissue stress of IR.

Translocation of AIF from mitochondria to the nucleus triggers caspase-independent cell death by inducing DNA damage (Yu et al., 2002; Sevrioukova, 2011; Natarajan and Becker, 2012). The mature form of AIF is anchored in the inner mitochondrial membrane (Ozaki et al., 2007; Chen et al., 2011). IR leads to a release of AIF from mitochondria into cytosol in isolated mouse heart, whereas administration of a calpain inhibitor prevents the loss of AIF from mitochondria (Chen et al., 2011). These results support that activation of mitochondrial localized μ-calpain is required to detach the AIF from the inner membrane (Ozaki et al., 2007; Chen et al., 2011) and indicate that retention of AIF within mitochondria provides cardioprotection during IR (Chen et al., 2011). The protection through retention of AIF within mitochondria can be due to a potential antioxidant role of the AIF or the prevention of AIF translocation to the nucleus. In the present study, mitochondria and nucleus were isolated from buffer perfused hearts to evaluate if IR increases the AIF translocation from mitochondria to nucleus. In the *in vivo* IR model, the length of the ischemic period was limited in that a relative long ischemic period could trigger a fatal arrhythmia occurrence in Hq mice (van Empel et al., 2005). Thus, a buffer perfused heart model was selected in the present study to avoid this confounding issue and to allow an ischemic period resulting in a moderate extent of mitochondrial and cardiac damage, relevant to *in situ* IR. Hq mice provide an experimental model to test the potential contribution of AIF to local mitochondrial protection compared to the deleterious cellular effects of nuclear translocation during IR.

METHODS

The experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees of Virginia Commonwealth University (VCU) and the McGuire Department of Veterans Affairs Medical Center.

PREPARATION OF MOUSE HEART FOR PERFUSION

Wild type or Hq mice [2–3 months of age (22–28 g), male] were anesthetized with pentobarbital sodium (100 mg/g i.p.) and anti-coagulated with heparin (1 IU/g i.p.) (Chen et al., 2011). In this study, only male mice were used because Hq mice had a gender-dependent response in an experimental stroke model (Yuan et al., 2009). Compared to male Hq mice, female Hq mice exhibited markedly decreased brain injury after experimental stroke (Yuan et al., 2009). Since the AIF gene is located on the X chromosome, using male mice will avoid gender-dependent gene dosage effects on cardioprotection. Hearts were excised and retrograde perfused via the aorta in the Langendorff mode (constant pressure, 75 mmHg) with modified Krebs-Henseleit buffer (composition, in mM: 115 NaCl, 4.0 KCl, 2.0 $CaCl_2$, 25 $NaHCO_3$, 1.1 $MgSO_4 \cdot H_2O$, 0.9 KH_2PO_4, and 5.5 glucose) oxygenated with 95% O_2+5% CO_2. Cardiac function was monitored with a balloon inserted into the left ventricle using Powerlab (AD Instruments, Colorado Springs, CO). Heart rate was maintained at 420 bpm with pacing during the equilibration period. Pacing was stopped during global ischemia and restored at 15 min reperfusion. In the IR group, hearts were buffer-perfused for 15 min, followed by 30 min global ischemia at 37°C within 30 min reperfusion (mitochondrial isolation) or 1 h reperfusion (infarction measurement). In the time control group, hearts were buffer-perfused without IR. Myocardial infarct size was determined using TTC staining (Chen et al., 2006). Coronary effluent was collected to determine LDH activity in each group (Chen et al., 2006).

ISOLATION OF CARDIAC MITOCHONDRIA FROM THE MOUSE HEART WITH PROTEASE

Trypsin was used in the mitochondrial isolation protocol in order to remove potential contamination from AIF located in the cytosol (Chen et al., 2011). The mouse heart was harvested and immediately placed in cold buffer A [100 mM KCl, 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 1 mM EGTA, 5 mM $MgSO_4$, 1 mM ATP]. Cardiac tissue was homogenized with a polytron at 10,000 rpm and incubated with trypsin (5 mg/g) for 15 min. Cold buffer B (0.2% BSA + buffer A) was then added into the homogenate. The homogenate was centrifuged at 500 × g for

10 min. The supernatant was centrifuged at 3000 × g for 10 min. to pellet mitochondria. The mitochondrial pellet was washed and suspended in 100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA.

ISOLATION OF NUCLEUS FROM BUFFER-PERFUSED MOUSE HEARTS

The polytron pellet was used to isolate nuclear components using a commercial kit from Thermo Scientific (Pittsburgh, PA, catalog # 78835) using supplied solutions and differential centrifugation according to instructions provided.

MEASUREMENT OF OXIDATIVE PHOSPHORYLATION AND ENZYME ACTIVITIES IN ISOLATED MITOCHONDRIA

Oxygen consumption by mitochondria was measured using a Clark-type oxygen electrode at 30°C using glutamate + malate (complex I substrate) or succinate + rotenone (complex II substrate) as donors (Lesnefsky et al., 1997). Respiratory enzyme activities [NADH-decylubiquinol oxidoreductase, rotenone sensitive (complex I)]; NADH ferricyanide oxioreductase (NFR, flavoprotein portion of complex I); Succinate-decylubiquinone oxidoreductase (complex II); and citrate synthase were measured in detergent solubilized mitochondria according to the method of Dr. Hoppel as previously described (Krahenbuhl et al., 1991; Lesnefsky et al., 1997; Chen et al., 2008).

DETECTION OF H_2O_2 PRODUCTION FROM ISOLATED MOUSE HEART MITOCHONDRIA

H_2O_2 production by isolated mitochondria was measured using the oxidation of the fluorogenic indicator Amplex red in the presence of horseradish peroxidase without exogenous SOD (Chen et al., 2003). Glutamate + malate and succinate + rotenone were used as complex I and complex II substrates, respectively. Rotenone and antimycin A were used to detect the maximal H_2O_2 generation from complex I and complex III, respectively.

DETERMINATION OF MITOCHONDRIAL CALCIUM RETENTION CAPACITY

Mitochondrial calcium retention capacity (CRC) was used to reflect opening of the mitochondrial permeability transition pore (MPTP) in isolated mitochondria (Chen et al., 2012a). CRC was studied in the single cell fluorimeter (PerkinElmer, Waltham, Massachusetts) using repetitive calcium pulses (Chen et al., 2012a). Freshly isolated mitochondria (0.25 mg) were incubated in buffer (150 mM sucrose, 50 mM KCl, 2 mM KPi, and 20 mM Tris/HCl, pH 7.4) for 90 s with stirring at 30°C with 0.5 μM calcium green. Succinate (5 mM) was used as substrate. Pulses of calcium (5 nmoles) were added at 60 s intervals. The number of pulses that resulted in calcium release indicated the onset of MPTP.

WESTERN BLOT ANALYSIS

Particle free cytosol, purified mitochondria, and nucleus were boiled for 5 min. in buffer including 4% (w/v) SDS, 1 mM 2-mercaptoethanol, 10 mM Tris/HCl (pH 6.8) and 10% (w/v) glycerol. Equal amounts of protein were loaded onto 4–15% or 4–20% SDS-PAGE (dependent on molecular weight of proteins), electrophoresed and transferred to a PVDF membrane. The membranes were first blocked by 5% non-fat milk for 1 h followed by exposure to primary antibodies overnight (Chen et al., 2011). Antibodies to AIF, PARP-1, GAPDH, lamin, and subunit IV of cytochrome oxidase were purchased from Cell Signaling Technology (Danvers, MA). Monoclonal PAR antibody was purchased from Millipore (Billerica, MA). The blots were incubated with peroxidase conjugated anti-rabbit or anti-mouse secondary antibody for 1 h prior to ECL detection (GE Healthcare Life Science, Pittsburgh, PA). The intensity of blotting was quantified by Fuji Film Image station (Edison, NJ).

STATISTICAL ANALYSIS

Data were expressed as the mean ± standard error of the mean. Differences among four groups were compared by one-way analysis of variance with *post-hoc* comparisons performed using the Student-Newman-Keuls test of multiple comparisons (*Sigmastat 3.5*, ProgramPaketet, Gothenburg, Sweden). Differences in CRC and H_2O_2 between wild type and Hq mice were compared by unpaired student *t*-test. A difference of $p < 0.05$ was considered significant.

RESULTS

IR DECREASED OXIDATIVE PHOSPHORYLATION IN BOTH WILD TYPE AND Hq HEART MITOCHONDRIA

There were no differences in the rate of oxidative phosphorylation between time control wild type and Hq mitochondria when glutamate + malate (complex I) or succinate + rotenone (complex II) were used as substrates. IR decreased the ADP-stimulated respiration in mitochondria from both wild type and Hq using either substrate (**Table 1**). The rate of dinitrophenol (DNP) uncoupled respiration was also decreased in both wild type and Hq mice following IR (**Table 1**), supporting that IR damages the electron transport chain. Interestingly, the rate of oxidative phosphorylation with glutamate + malate in Hq hearts was decreased following IR compared to corresponding wild type (**Table 1**), whereas the rate of succinate oxidation was similar in Hq and wild type hearts following IR (**Table 1**). These results indicated that

Table 1 | The rate of oxidative phosphorylation in wild type and Hq mitochondria following IR.

Mice	*n*	State 3	State 4	RCR	ADP/O	DNP
GLUTAMATE + MALATE WAS USED AS COMPLEX I SUBSTRATE (nAO/min/mg PROTEIN)						
WT-TC	$N = 8$	324 ± 11	56 ± 5	6.2 ± 0.8	2.73 ± 0.108	341 ± 18
WT-IR	$N = 8$	271 ± 10*	56 ± 4	5.1 ± 0.5	2.80 ± 0.09	281 ± 11*
Hq-TC	$N = 6$	315 ± 27	51 ± 9	6.7 ± 0.8	2.80 ± 0.08	321 ± 40
Hq-IR	$N = 7$	224 ± 12*†	52 ± 9	5.3 ± 1.1	2.76 ± 0.13	229 ± 16*†
SUCCINATE + ROTENONE WAS USED AS COMPLEX II SUBSTRATE (nAO/min/mg PROTEIN)						
WT-TC	$N = 8$	474 ± 14	144 ± 3	3.3 ± 0.1	2.00 ± 0.07	435 ± 12
WT-IR	$N = 8$	266 ± 11*	133 ± 6	2.0 ± 0.1*	1.84 ± 0.05	339 ± 14*
Hq-TC	$N = 6$	496 ± 20	133 ± 18	3.4 ± 0.2	2.08 ± 0.06	471 ± 30
Hq-IR	$N = 7$	268 ± 15*	124 ± 6	2.2 ± 0.1*	2.07 ± 0.09	346 ± 25*

*Mean ± s.e.m. *$p < 0.05$ vs. corresponding time control (TC); †$p < 0.05$ vs. WT-IR (ischemia-reperfusion). RCR, respiratory control ratio. DNP (0.3 mM), dinitrophenol to measure the rate of uncoupled respiration.*

IR led to additional decreases in respiration in Hq mouse heart mitochondria when NADH-dependent substrates were used.

IR DECREASED COMPLEX I ACTIVITY IN WILD TYPE BUT NOT Hq MOUSE HEART MITOCHONDRIA

In order to test if IR led to further damage to complex I; NADH:decylubiquinol oxidoredutase, NFR, and complex II activities were measured. Complex I activity [shown as the ratio of complex I/CS (citrate synthase), **Table 2**] was decreased in wild type following IR compared to time control. However, complex I activity was not decreased in Hq mitochondria following IR compared to its corresponding time control. The NADH dehydrogenase activity (NFR) was not decreased in either wild type or Hq mice following IR (**Table 2**), consistent with previous study (Chen et al., 2007; Szczepanek et al., 2011). NADH dehydrogenase activity was slightly higher in Hq mouse heart mitochondria following IR compared to corresponding wild type (**Table 2**). The physiological significance of this subtle difference is unclear. IR did not alter complex II activity in wild type or Hq mice (**Table 2**). These results support that IR leads to a complex I defect in wild type mouse heart mitochondria.

THE GENERATION OF H_2O_2 WAS DECREASED IN Hq MOUSE HEART MITOCHONDRIA FOLLOWING IR

There were no differences in H_2O_2 generation between time control wild type and Hq heart mitochondria using complex I (**Figure 1A**) or complex II substrates (**Figure 1C**). IR markedly increased the production of H_2O_2 in wild type but not in Hq mouse heart mitochondria with either a complex I or complex II substrate (**Figures 1A,C**). Compared to time control, inhibition of complex I using rotenone dramatically increased H_2O_2 generation in wild type mouse heart following IR (**Figure 1B**). In contrast, rotenone inhibition did not increase the H_2O_2 generation in Hq mouse heart following IR (**Figure 1B**). The maximal ROS generation from mitochondria was induced with antimycin A inhibition. Inhibition of complex III using antimycin A increased the H_2O_2 generation in both wild type and Hq mouse heart following IR vs. time control (**Figure 1D**). However, there were no differences in ROS generation between wild type and Hq mice with or without IR.

CARDIAC INJURY WAS DECREASED IN Hq MOUSE HEART FOLLOWING IR

In the buffer perfused hearts, myocardial injury was decreased in the Hq mouse heart following IR compared to wild type. Knock down of AIF content in Hq mouse heart did not affect the cardiac function before ischemia (**Figure 2A**). IR decreased left ventricular developed pressure (LVDP) in both wild type and Hq hearts vs. time control. Systolic function was improved in Hq hearts vs. wild type during reperfusion (**Figure 3A**). Strikingly, the infarct size was also much smaller in Hq mice than in wild type (**Figure 2B**). The release of LDH into coronary effluent was much lower in Hq mice than in wild type (**Figure 2C**), also indicating less necrosis.

IR DECREASED THE CRC IN BOTH WILD TYPE AND Hq MOUSE HEART MITOCHONDRIA

The CRC (**Figure 3A**) was decreased in mitochondria from non-ischemic Hq mice compared to wild type (**Figure 3B**), suggesting that a decrease in AIF content within mitochondria sensitizes to calcium-stimulated MPTP opening. Although there was a slight difference in the CRC between Hq and wild type following IR (**Figures 3A,B**), the small magnitude of this difference may not exert a significant impact on cardiac injury during IR.

IR ACTIVATED PARP-1 IN WILD TYPE BUT NOT IN Hq MICE

Activation of PARP-1 during IR increases the generation of PAR [Poly (ADP-ribose) (PAR)] that is transferred to cytosol and mitochondria (Sevrioukova, 2011). The content of PAR was markedly increased in wild type following IR compared to time control (**Figures 4A–C**), indicating that IR leads to PARP-1 activation. In contrast, IR did not alter the PAR content in Hq mice following IR (**Figures 4A–C**).

IR DECREASED THE AIF CONTENT WITHIN MITOCHONDRIA AND INCREASED NUCLEAR AIF CONTENT IN WILD TYPE MICE

The precursor of AIF (67 kd) is nuclear-encoded and subsequently transported into the mitochondrial matrix via its mitochondrial targeting sequence (Sevrioukova, 2011). The mature form of AIF (62 kd) is formed in the matrix through cleavage of precursor protein via a mitochondrial matrix peptidase (Sevrioukova, 2011). The mature AIF (62 kd) is transferred into the mitochondrial intermembrane space through the Tim23 protein (Sevrioukova, 2011). Consistent with these concepts, two AIF bands are detected in non-ischemic wild type mouse heart mitochondria (**Figure 4D**). In contrast, the mature form of AIF (62 kd) is almost undetectable in Hq mouse heart mitochondria (**Figure 4D**), confirming the lower content of AIF within mitochondria. The content of AIF within mitochondria was decreased in wild type mice following IR compared to time control (**Figure 4D**). The AIF content (62 kd) in nucleus was increased in wild type hearts following IR (**Figure 4E**).

Table 2 | The enzyme activities of Complex I and II in wild type and Hq mitochondria with and without IR.

Mice	*n*	CS	Complex I/CS	NFR/CS	CII/CS	CII+Q/CS
WT-TC	$N = 8$	2934 ± 1.4	0.200 ± 0.019	0.656 ± 0.055	0.039 ± 0.011	0.111 ± 0.020
WT-IR	$N = 8$	2937 ± 1.9	0.142 ± 0.010*	0.542 ± 0.029	0.059 ± 0.008	0.142 ± 0.012
Hq-TC	$N = 6$	2751 ± 1.7	0.195 ± 0.019	0.767 ± 0.050	0.045 ± 0.010	0.132 ± 0.026
Hq-IR	$N = 7$	2545 ± 1.7	0.174 ± 0.019	$0.793 \pm 0.081^{\dagger}$	0.039 ± 0.010	0.103 ± 0.027

*Mean ± s.e.m. *$p < 0.05$ vs. WT-TC; $^{\dagger}p < 0.05$ vs. WT-IR. NFR, NADH dehydrogenase; CS, citrate synthase. Complex II activity (CII) was determined in the presence and absence of exogenous decylubiquinone (Q).*

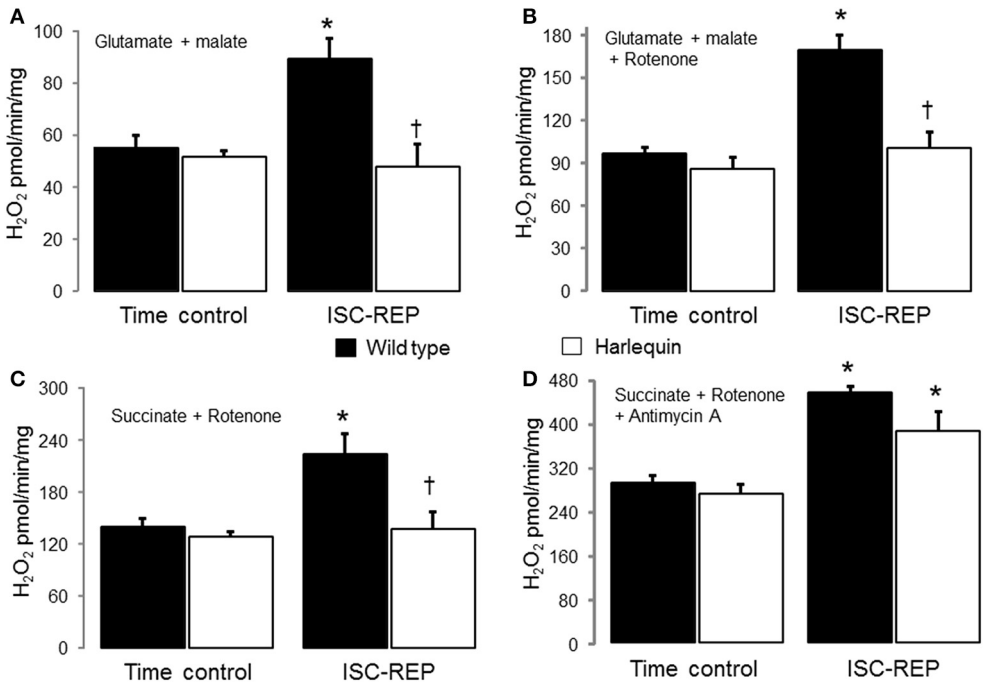

FIGURE 1 | Ischemia-reperfusion (IR) increases the net H_2O_2 production in wild type but not in Hq mouse cardiac mitochondria. There were no differences in the net release of H_2O_2 between wild type and Harlequin mouse heart mitochondria from non-ischemic hearts. IR increased the generation of H_2O_2 in wild type but not in Harlequin mice compared to time control **(A)**. The maximal H_2O_2 generation from complex I was measured using rotenone to inhibit complex I. An AIF deficiency in Harlequin mice did not alter the maximal H_2O_2 generation from complex I compared to wild type in control heart **(B)**. The maximal H_2O_2 generation from complex I was also decreased in Harlequin mice following IR compared to wild type **(B)**. Knock down of AIF in Harlequin mice did not alter the H_2O_2 generation using succinate + rotenone as complex II substrates compared to wild type control. The H_2O_2 generation was decreased in Harlequin mice following IR compared to wild type **(C)**. The maximal H_2O_2 generation from complex III in the presence of antimycin A was not decreased in Harlequin mice following IR compared to wild type **(D)**. Data are expressed as mean ± s.e.m.; $^*p < 0.05$ vs. time control; $^\dagger p < 0.05$ vs. wild type IR.

DISCUSSION

In the present study, a deficiency of AIF within Hq mouse heart mitochondria does not increase the net release of H_2O_2 compared to wild type, consistent with reports in brain mitochondria. IR leads to increased net release of ROS from wild type heart mitochondria compared to non-ischemic controls. In contrast, the net release of ROS from Hq heart mitochondria is unchanged following IR. Thus, genetic knockdown of AIF within mitochondria does not increase the net release of ROS from the electron transport chain. These findings suggest that AIF less likely functions as a key mitochondrial antioxidant in the heart, especially following the stress of IR. The PARP-1 is activated in wild type but not in Hq mice following IR. Translocation of the AIF from mitochondria to the nucleus is increased in wild type but not in Hq mice following IR. The decrease in cardiac injury in Hq mouse heart accompanied by less AIF translocation to the nucleus suggests that the amount of AIF that relocates to the nucleus, rather than the AIF content within mitochondria, is the key factor that contributes to cardiac injury during IR.

COMPLEX I DAMAGE DURING IR

Complex I activity is decreased in heart mitochondria following both *in vivo* (Rouslin and Millard, 1980; Rouslin, 1983) and *in vitro* IR (Lesnefsky et al., 2001; Gustafsson and Gottlieb, 2008; Murphy and Steenbergen, 2008). In the present study, IR leads to decreased complex I activity without alteration in NADH dehydrogenase activity (NFR). These results indicate that ischemia likely damages complex I at the iron sulfur centers (Chen et al., 2008) distal to the flavoprotein, in line with previous studies (Ohnishi and Trumpower, 1980; Chen et al., 2006; Zhou et al., 2006; Szczepanek et al., 2011).

Oxidative modification of complex I by nitrosation (Burwell et al., 2006) or glutathionylation (Hurd et al., 2008) or the modification of its inner membrane environment via depletion of cardiolipin (Paradies et al., 2004) all contribute to decreases in activity. Mitochondrial AIF content also affects complex I activity, especially in brain and retina (Klein et al., 2002; van Empel et al., 2005). Depletion of AIF also decreases complex I activity in heart mitochondria (Pospisilik et al., 2007). However, the effect of lower expression of AIF in Hq mice on heart mitochondrial complex I activity is not consistent (Szczepanek et al., 2013). Thus, an AIF deficiency may affect complex I activity in a tissue-dependent manner. Genetic depletion of PARP-1 protects complex I activity in mouse heart following IR (Zhou et al., 2006), indicating that PARP-1 activation contributes to the complex I defect during IR. In the present study, IR decreases complex I activity accompanied by an activated PARP-1 in wild type mice. In contrast, IR does not decrease complex I activity in Hq mice. PARP-1 is

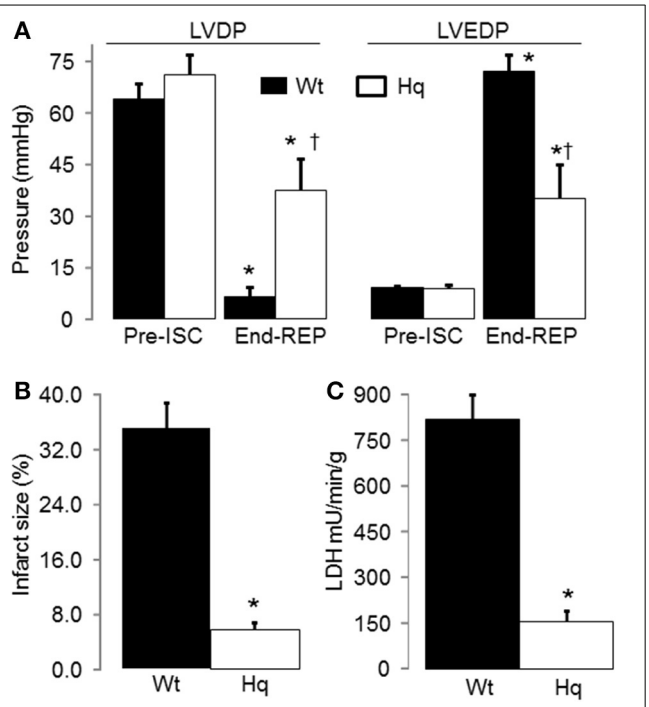

FIGURE 2 | IR led to decreased cardiac injury in Harlequin mice compared to wild type. Heart rate was maintained at 420 bpm by pacing during 15 min equilibration perfusion. The pacing was stopped during ischemia and resumed at 15 min reperfusion. The recovery of myocardial contractile function during reperfusion, shown by the improvement in left ventricular developed pressure (LVDP mmHg, **A**), was improved in Harlequin mice compared to wild type. The diastolic function was also improved in Harlequin mice following IR, reflected by a decrease in left ventricular end-diastolic pressure (LVEDP) at the end of reperfusion **(B)**. The cardiac injury during IR was decreased in Harlequin mice as shown by a smaller infarct size **(C)** and less LDH release into coronary effluent. Data are expressed as mean ± s.e.m.; $^{*}p < 0.05$ vs. time control (TC); $^{\dagger}p < 0.05$ vs. wild type untreated IR.

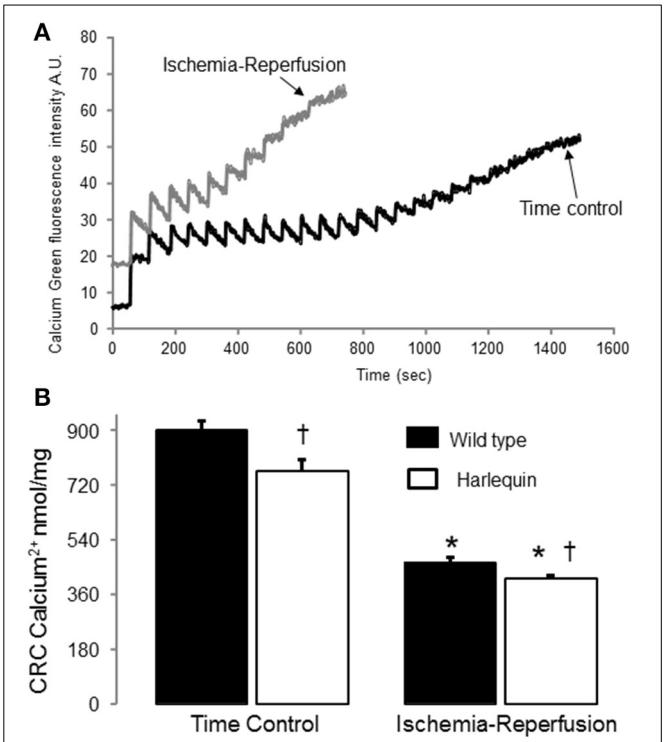

FIGURE 3 | IR decreased the CRC in wild type and Harlequin cardiac mitochondria. Original tracings of CRC measurement in wild type heart mitochondria were shown in panel **(A)**. The CRC was decreased in control Harlequin mouse heart mitochondria compared to wild type **(B)**, suggesting that the sensitivity to MPTP opening was increased in Harlequin mice in the basal condition. IR led to decreased CRC in both wild type and Harlequin mice compared to time control **(B)**. Compared to wild type, IR led to a slight further decrease in CRC in Harlequin mice **(B)**. Data are expressed as mean ± s.e.m.; $^{*}p < 0.05$ vs. time control; $^{\dagger}p < 0.05$ vs. wild type IR.

also not activated in Hq mice following IR. These results support that activation of PARP-1 contributes to the complex I defect during IR. Since PARP-1 is considered as a nuclear protein, complex I inhibition by PARP-1 activation appears to present a challenge (**Figure 5**) (Zhou et al., 2006). Recently, a mitochondrial localized PARP-1 has been identified (Rossi et al., 2009). Thus, activation of PARP-1 may directly regulate complex I activity (**Figure 5**).

COMPLEX I INHIBITION AND CARDIOPROTECTION DURING IR

The ischemia-damaged respiratory chain including complex I, is a key source of ROS that increases cardiac injury (Turrens, 2003; Chen et al., 2007). Blockade of proximal electron transport using amobarbital before ischemia protects complex I and decreases cardiac injury during reperfusion, supporting that preservation of complex I activity reduces cardiac injury (Chen et al., 2006). Transient partial (Xu et al., 2014) or complete (Stewart et al., 2009; Chen et al., 2012b) blockade of complex I at the onset of reperfusion decreases myocardial injury in buffer perfused hearts, indicating that a temporary complex I inhibition is also beneficial for cardiac recovery. However, persistent, severe complex I inhibition is detrimental to the heart both at baseline and for recovery during reperfusion (Karamanlidis et al., 2013). In contrast to wild type, the complex I activity is not altered in Hq mice following IR. The lack of damage to complex I during IR in Hq mice may lead to decreased ROS generation that contributes to the observed decrease in cardiac injury in Hq mice. Alteration of mitochondrial antioxidants including thioredoxin reductase-2 significantly affects a release of H_2O_2 from mitochondria. The IR-induced complex I damage may also increase H_2O_2 by inhibiting thioredoxin reductase-2 through alteration of its redox state (Rigobello et al., 2006; Horstkotte et al., 2011; Stanley et al., 2011). The increased oxidative stress will favor activated μ-calpain to cleave AIF and facilitate its release from mitochondria (Norberg et al., 2010).

TRANSLOCATION OF AIF FROM MITOCHONDRIA TO NUCLEUS INCREASES CARDIAC INJURY DURING IR

The mature form of AIF (62 kd) is anchored at the inner mitochondrial membrane within the intermembrane space (Otera et al., 2005). Release of AIF from the mitochondria and translocation to the nucleus to activate caspase-independent cell death is a multistep process. First, the mature AIF bound within mitochondria on the inner membrane requires liberation. Cleavage of

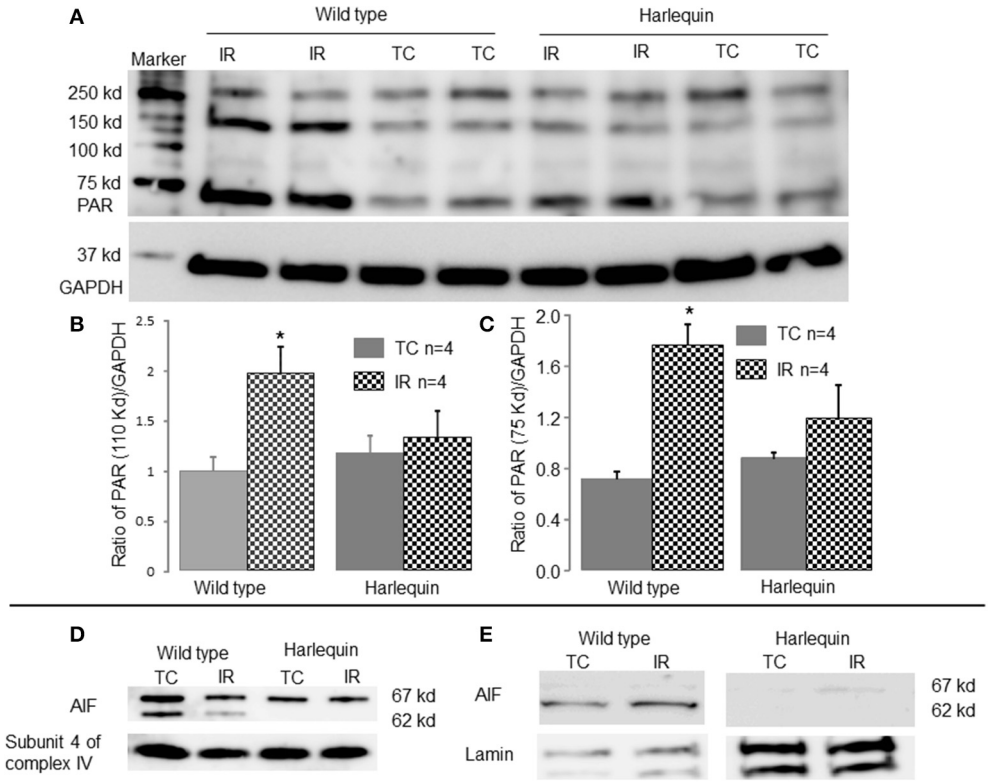

FIGURE 4 | IR increases AIF translocation from mitochondria to nucleus in wild type. The content of poly (ADP-ribose) (PAR) was markedly increased in wild type mice following IR compared to time control, indicating that IR activated PARP-1 **(A–C)**. There were no differences in the PAR content in Hq mice between time control and mice following IR **(A–C)**. GAPDH was used as a cytosol marker for loading control. The precursor form of AIF (67 kd) and the mature form of AIF (62 kd) were detected in wild type mouse heart mitochondria **(D)**. Compared to control, IR markedly decreased the content of the mature form of AIF (62 kd band) in wild type mice **(D)**. The mature AIF content in Harlequin mice was of course decreased compared to wild type **(D)**. In wild type, IR increased AIF content (62 kd) in nucleus compared to time control, indicating a translocation of AIF from mitochondria to nucleus **(E)**. Subunit 4 of cytochrome oxidase and lamin were used as protein loading control for mitochondria and nucleus, respectively.

the mature 62 kd form of AIF by activated mitochondrial calpains (Ozaki et al., 2007), *t*-bid (Cabon et al., 2012) or other proteases can liberate AIF from the inner membrane, with release of a truncated, approximately 57 kd AIF peptide. Next, permeation of the outer mitochondrial membrane is required for AIF release (Ozaki et al., 2007). IR increases MPTP opening as a mechanism of increased outer membrane permeability (Weiss et al., 2003), with oxidative stress is a key contributor to the increased susceptibility to MPTP opening during IR (Weiss et al., 2003; Halestrap et al., 2004; Chen et al., 2012a). In contrast, activation of PARP-1 is not involved in the permeation of the outer mitochondrial membrane during IR (Schriewer et al., 2013). The decreased AIF content in the purified mitochondria following IR supports that IR does lead to a loss of AIF from mitochondria. The increased MPTP opening in wild type mice during reperfusion favors a release of AIF from mitochondria into cytosol with subsequent translocation to the nucleus. It currently appears that even following MPTP, the released AIF is the 57 kd cleaved form, at least based upon calcium activation of mitochondrial calpains concomitant with MPTP. This area of calcium mediated injury deserves further consideration. As discussed above, IR activates PARP-1 in wild type but not Hq mice (Pacher and Szabo, 2007). Although activation of PARP-1 provides a beneficial effect to repair DNA damage, over activation of PARP-1 has a detrimental effect via consumption of NAD^+ (Pacher and Szabo, 2007). PAR, which is generated by activation of PARP-1 within nucleus, is released into cytosol and subsequently relocates to mitochondria to induce AIF release from mitochondria (Pacher and Szabo, 2007). Interestingly, a portion of the mature AIF is also reported to be loosely attached on the mitochondrial outer membrane (Yu et al., 2006, 2009). The PAR can detach the AIF from the outer membrane (**Figure 5**) (Wang et al., 2009). Outer membrane bound-AIF has been identified in mouse heart mitochondria (Chen and Lesnefsky, unpublished data). Thus, activation of PARP-1 may increase AIF translocation to the nucleus through detachment of mature AIF from the outer membrane, in addition to release of the pool from the inner membrane via cleavage. The accumulation of AIF in the nucleus accompanied by increased cardiac injury in wild type mice following IR supports the proposal that the translocation of AIF from the mitochondria to the nucleus augments cardiac injury.

GENETIC INHIBITION OF AIF EXPRESSION IN Hq MICE DECREASES CARDIAC INJURY DURING IR

Although cardiac injury is increased in Hq mouse heart following *in vivo* IR compared to wild type controls (van Empel et al., 2005), cardiac injury is actually decreased in Hq mouse heart

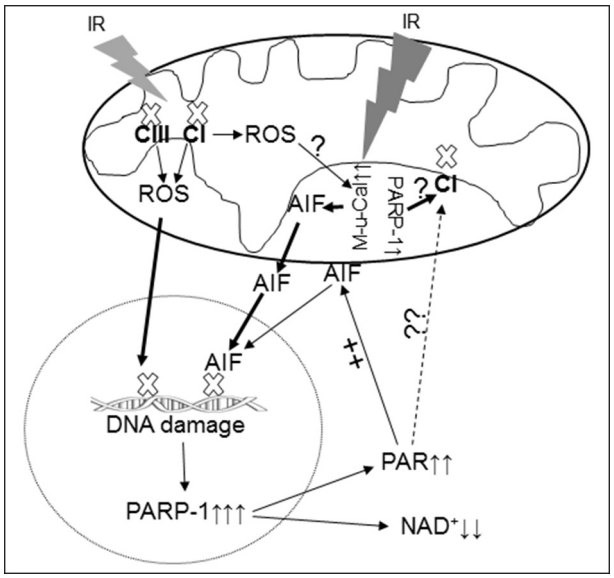

FIGURE 5 | Depiction of the PARP-1 activation and AIF translocation during IR. IR damages the electron transport chain and increases the ROS generation from complex I and complex III and activates mitochondrial μ-calpain (M-μ-Cal) to induce release of AIF from the inner mitochondrial membrane. Translocation of AIF from mitochondria to nucleus enhances DNA damage and PARP-1 activation, in turn further reducing NAD^+ content. Oxidative stress during IR leads to DNA damage DNA that activates the PARP-1 that leads to decreased NAD^+ content in cytosol and increased generation of PAR within nucleus. The PAR is released into cytosol and relocates to mitochondria and facilitates AIF release from the outer mitochondrial membrane. The activated PARP-1 may contribute to complex I defect through an indirect mechanism. IR may activate mitochondrial localized PARP-1 to induce direct complex I damage in cardiac mitochondria. CI, complex I; CIII, complex III; M-μ-Cal, mitochondrial μ-calpain.

following *in vitro* IR. Several key differences likely contribute to these divergent results. In the present study, only male Hq mice were used based upon the rationale discussed in Methods, whereas both female and male Hq mice were included in the previous *in vivo* study (van Empel et al., 2005). This is important, since gender-related cardiac protection as well as gene dosage issues related to the X chromosome location of the *aif* gene can introduce variability. In the present study, only 2–3-month-old mice were used whereas middle-aged and elderly mice were used in the *in vivo* study (van Empel et al., 2005). *In vivo*, there is the additional impact of exogenous inflammatory cells (with or without AIF deficiency). Furthermore, *in vivo*, substrate utilization is uncontrolled. The metabolism of fatty acids *in vivo*, in contrast to glucose utilization in the current study, may have exacerbated the phenotype of mitochondrial defects present. Taken together, gender, age, exogenous cells and the different IR models likely resulted in the differences observed in our current study compared to the previous *in vivo* study. An isolated heart was used in order to focus on myocyte specific responses in the present study.

In summary, the key contribution of AIF to cardiac injury during IR is related to release from mitochondria and activation of programmed cell death via cytosolic transport, nuclear import and DNA cleavage. The findings in cardiac mitochondria from Hq mice compared to littermate controls support that a decreased content of AIF does not enhance ROS production from mitochondria nor augment cardiac injury at baseline nor during IR. Thus, AIF does not exert significant mitochondrial antioxidant protection during IR. The prevention of AIF translocation to nucleus is a potentially powerful approach to reduce cardiac injury.

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Differential effects of buffer pH on Ca^{2+}-induced ROS emission with inhibited mitochondrial complexes I and III

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Excessive mitochondrial reactive oxygen species (ROS) emission is a critical component in the etiology of ischemic injury. Complex I and complex III of the electron transport chain are considered the primary sources of ROS emission during cardiac ischemia and reperfusion (IR) injury. Several factors modulate ischemic ROS emission, such as an increase in extra-matrix Ca^{2+}, a decrease in extra-matrix pH, and a change in substrate utilization. Here we examined the combined effects of these factors on ROS emission from respiratory complexes I and III under conditions of simulated IR injury. Guinea pig heart mitochondria were suspended in experimental buffer at a given pH and incubated with or without $CaCl_2$. Mitochondria were then treated with either pyruvate, a complex I substrate, followed by rotenone, a complex I inhibitor, or succinate, a complex II substrate, followed by antimycin A, a complex III inhibitor. H_2O_2 release rate and matrix volume were compared with and without adding $CaCl_2$ and at pH 7.15, 6.9, or 6.5 with pyruvate + rotenone or succinate + antimycin A to simulate conditions that may occur during *in vivo* cardiac IR injury. We found a large increase in H_2O_2 release with high [$CaCl_2$] and pyruvate + rotenone at pH 6.9, but not at pHs 7.15 or 6.5. Large increases in H_2O_2 release rate also occurred at each pH with high [$CaCl_2$] and succinate + antimycin A, with the highest levels observed at pH 7.15. The increases in H_2O_2 release were associated with significant mitochondrial swelling, and both H_2O_2 release and swelling were abolished by cyclosporine A, a desensitizer of the mitochondrial permeability transition pore (mPTP). These results indicate that ROS production by complex I and by complex III is differently affected by buffer pH and Ca^{2+} loading with mPTP opening. The study suggests that changes in the levels of cytosolic Ca^{2+} and pH during IR alter the relative amounts of ROS produced at mitochondrial respiratory complex I and complex III.

Keywords: mitochondrial complex I, mitochondrial complex III, reactive oxygen species, simulated ischemia, mitochondrial permeability transition pore, Ca^{2+}, pH

INTRODUCTION

Ischemic injury is a multifactorial process that predisposes to further injury during reperfusion (Camara et al., 2007; Aldakkak et al., 2008a,b, 2011). One key aspect of ischemic injury is the increase in mitochondrial production of reactive oxygen species (ROS) above the antioxidant ability of the endogenous ROS scavenging system (Trachootham et al., 2008; Stowe and Camara, 2009; Camara et al., 2010). Under normal conditions, ROS emission is maintained at a low level, which is important for regular cellular function. But an increase in ROS emission (more production and less scavenging) during ischemia leads to oxidative stress and apoptosis that leads to cellular dysfunction and death (Trachootham et al., 2008).

It is widely acknowledged that the main sources of ROS production (mainly superoxide, $O_2^{\bullet-}$) during cardiac ischemia and reperfusion (IR) injury are respiratory complex I and complex III of the mitochondrial electron transport chain (ETC) (Chen et al., 2007, 2008; Kim et al., 2012; Musatov and Robinson, 2012); but it is unclear what is the relative contribution of complexes I and III on enhancing ROS generation during IR and when ROS scavenging systems fail. Unlike the other components of the ETC, these complexes are more prone to electron leak, even under physiologic conditions due to their electron transfer mechanisms (Musatov and Robinson, 2012). Thus, during IR, these complexes are prone to self-induced oxidative damage (Gadicherla et al., 2012), which impairs their activity, predisposing them to even greater ROS production (Rouslin, 1983; Chen et al., 2007, 2008; Musatov and Robinson, 2012). The damage to complex I may occur abruptly within 20 min of ischemia, whereas damage to complex III may occur more gradually as ischemia proceeds (Rouslin, 1983; Chen et al., 2007).

We showed previously in a guinea pig model of *ex vivo* global IR injury two distinct time-dependent phases of ROS emission during ischemia; an early phase of low/moderate accumulation of ROS, and a late phase of high ROS accumulation, followed by a surge of ROS during early reperfusion (Kevin et al., 2003; Camara et al., 2007; Aldakkak et al., 2008a,b, 2011). This two-phase release of ROS may correspond with the timing of damage of complexes I and III as reported by others (Chen et al., 2007). To understand this, we investigated in a recent isolated mitochondrial study the impact of extreme conditions that might mimic the period of ischemia and reperfusion on ROS emission (Aldakkak et al., 2013). We found a large increase in hydrogen peroxide (H_2O_2) release when complex III electron transfer was blocked by antimycin A (AA) in succinate-energized mitochondria incubated in elevated extra-matrix Ca^{2+} buffer. However, these studies did not evaluate the impact of changes in buffer pH and mitochondrial permeability transition pore (mPTP) opening by excess Ca^{2+} overload, both of which are important modulating factors that can occur in mitochondria during IR and contribute to ROS production.

During cardiac ischemia, cytosolic pH levels decrease (Park et al., 1999), due in part to increased lactate production via anaerobic glycolysis, and cytosolic Ca^{2+} levels rise (Aldakkak et al., 2011), due in part to reduced Ca^{2+} sequestration by the sarcoplasmic reticulum. Mitochondrial Ca^{2+} levels increase (Aldakkak et al., 2008a); in part as a result of the increase in uptake by the mitochondrial Ca^{2+} uniporter. Matrix pH levels, however, depend on many factors including cytosolic pH, mitochondrial Na^+/H^+, K^+/H^+, and Na^+/Ca^{2+} exchange, proton (H^+) leak, and variable H^+ pumping rates by complexes I, III, IV, and V. In the first 5 min of cardiac ischemia, cytosolic pH in affected cardiomyocytes has been reported to drop 0.5 pH units from the initial pH around 7.15, and eventually to reach as low as 6.0 with a longer ischemia time (Stamm et al., 2003; Murphy and Steenbergen, 2008). Interestingly, one study (Selivanov et al., 2008) demonstrated that pH can directly modulate ROS production from the ETC; they reported that an alkaline pH increased formation of $O_2^{\bullet-}$ due to increased stabilization of the semiquinone radical in the Q cycle of complex III (Selivanov et al., 2008). However, as cardiac ischemia progresses there is a gradual decrease in cellular pH (Park et al., 1999) and a gradual increase in ROS levels (Vanden Hoek et al., 1997; Becker et al., 1999; Kevin et al., 2003), indicating that factors other than just pH alone are involved.

The aim of our study was to investigate the combined effects of pH and elevated Ca^{2+} on the rate of release of H_2O_2 from mitochondria, using substrates and inhibitors of respiratory complexes, in an attempt to mimic cardiac IR. Specifically, we looked at the effect of acidic pH and high Ca^{2+} using two different combinations of mitochondrial substrate + inhibitor conditions. We utilized pyruvate + rotenone (ROT) to mimic abundance of pyruvate with impaired complex I, or succinate + AA to mimic abundance of succinate with impaired complex III (Turrens and Boveris, 1980; Kakinuma et al., 1994; Starkov et al., 2004). The elevation in Ca^{2+} was to induce mitochondrial matrix Ca^{2+} overload sufficient to induce mPTP opening to mimic an effect of cardiac IR injury. We hypothesized that a decrease in pH, to further mimic conditions of ischemia, would additionally modulate $O_2^{\bullet-}$ generation from complex III and/or complex I as assessed by H_2O_2 release in isolated mitochondria.

Mitochondrial inhibitors of complexes I-V can cause either reduced or enhanced $O_2^{\bullet-}$ generation depending on their site of action (Becker et al., 1999). With pyruvate as the substrate, we chose ROT because it inhibits transfer of electrons from iron-sulfur (Fe-S) centers in complex I at the binding site for quinol, thus creating a backup of electrons and a highly reduced NADH pool. This scenario would mimic IR-induced damage to complex I proteins to cause impaired electron transfer via Fe-S centers (Gadicherla et al., 2012). Studies have shown that selective accumulation of succinate is a universal metabolic signature of ischemia in the heart and is responsible for mitochondrial ROS production during reperfusion (Lukyanova, 2013; Chouchani et al., 2014). Therefore, with succinate as the substrate, we chose AA because it inhibits the quinone -reducing center (Q_i) of complex III to prevent the semiquinone radical formed at the Q_o site from being oxidized. Since this impedes electron transfer to the Q_i site, the semiquinone can then transfer its singlet electron to O_2 to produce $O_2^{\bullet-}$ at complex III (Starkov and Fiskum, 2001). With succinate, AA can also enhance $O_2^{\bullet-}$ generation at complex I. This scenario mimics damage to complex I plus damage to the Fe-S peptide of complex III during ischemia when quinol oxidation at the Q_o site is limited; this leads to "bypass reactions" that enhance $O_2^{\bullet-}$ generation at that site (Lesnefsky et al., 2001; Muller et al., 2002). We chose not to block complex IV, which would mimic diminished O_2 levels during ischemia, because in this case it would prevent $O_2^{\bullet-}$ generation at complex III like the complex III Q_o site inhibitor myxothiazol, while stimulating $O_2^{\bullet-}$ generation at complex I (Turrens et al., 1985).

MATERIALS AND METHODS

All experiments were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

MITOCHONDRIA ISOLATION

Heart mitochondria were isolated from ketamine-anesthetized (50 mg/kg ip) guinea pigs (250–350 g) as described previously (Gadicherla et al., 2012; Aldakkak et al., 2013; Blomeyer et al., 2013). Briefly, ventricles were excised, placed in an isolation buffer (buffer A) that contained (in mM) 200 mannitol, 50 sucrose, 5 KH_2PO_4, 5 MOPS, 1 EGTA, and 0.1% bovine serum albumin (BSA; all chemicals from Sigma, St. Louis, MO, USA), with pH adjusted to 7.15 with KOH. Ventricles were then minced into 1-mm^3 pieces. The suspension was homogenized in isolation buffer containing 5U/ml protease (Bacillus licheniformis; Sigma), followed by differential centrifugation at 4°C, and the final pellet was resuspended in isolation buffer and kept on ice. Protein content was determined by the Bradford method. Mitochondrial suspension was adjusted to yield 12.5 mg protein/ml for experimental purpose. Details of the experimental approach are provided in the Supplementary Material.

EXPERIMENTAL PROTOCOL

Experiments were conducted at room temperature (25°C), with mitochondria (0.5 mg protein/ml) suspended in experimental buffer (buffer B) that contained (in mM) 130 KCl (EMD Chemicals, Gibbs-town, NJ, USA), 5 K_2HPO_4, 20 MOPS, 0.001 $Na_4P_2O_7$, and 0.1% BSA. This assured that only 40 μM EGTA was carried over from the isolation buffer (buffer A) into the experimental buffer. Based on the experimental protocol and conditions, the buffer pH was specifically adjusted upward from 6.5 to 6.9 and 7.15 by adding KOH. The respiration buffer contained 0 or 150 μM $CaCl_2$; concentrations of $CaCl_2$ between 20 and 60 μM had no significant effects on H_2O_2 production (Figure S.3) and 100 μM $CaCl_2$ gave inconsistent data, so these data are not reported. From the residual EGTA concentration of 40 μM, we estimated 150 μM $CaCl_2$ to be equivalent to ≈ 220 nmol $CaCl_2$/mg protein. After adding $CaCl_2$ (or H_2O), 10 mM Na^+ pyruvate or Na^+ succinate (Sigma) was added. Then either complex I blocker ROT (10 μM; Sigma) or complex III blocker AA (5 μM; Sigma) was added.

MITOCHONDRIAL FLUORESCENCE MEASUREMENTS

Mitochondria were suspended in buffer B in a 1 ml cuvette inside a spectrophotometer (QM-8; Photon Technology International (PTI), Birmingham, NJ, USA). The rate of H_2O_2 release was measured using Amplex red (12.5 μM; Molecular Probes, Eugene, OR, USA) and horseradish peroxidase (0.1 U/ml; Sigma) at excitation and emission wavelengths of 530 and 583 nm, respectively. H_2O_2 is the direct product of $O_2^{\bullet-}$ when catalyzed by $O_2^{\bullet-}$ dismutase (SOD) in the absence of nitric oxide. H_2O_2 levels were calibrated over a range of 10–200 nM H_2O_2 (Sigma) added to buffer B in the absence of mitochondria and in the presence of Amplex red and horseradish peroxidase. Mitochondrial volume change (increase/decrease) was assessed by monitoring changes in 90° light scattering at an excitation and emission wavelength of 520 nm inside the same cuvette-based PTI.

MITOCHONDRIAL O_2 CONSUMPTION

Oxygen consumption was measured polarographically using a respirometry system (System S 200A; Strathkelvin Instruments, Glasgow, Scotland). Respiration experiments using pyruvate or succinate at pH 7.15 and without Ca^{2+} were initially conducted to determine the viability of mitochondria for the rest of the experiments. Respiration was initiated by adding 10 mM complex I substrate Na^+ pyruvate or the complex II substrate Na^+ succinate. State 3 respiration was measured after adding 250 μM ADP (Sigma), and state 4 respiration was measured after complete phosphorylation of the added ADP. The respiratory control index (RCI) was calculated as the ratio of the rate of state 3 to state 4 respiration. Only mitochondria with an RCI of 10 or above with pyruvate or an RCI of 3 or above with succinate were used in the experiments. To assess the effects of pH and extra-matrix (e) $[Ca^{2+}]_e$ on O_2 consumption (respiration), we added either H_2O (control) or $CaCl_2$ for a final concentration of 150 μM to the mitochondrial suspension at three pHs (7.15, 6.9 or 6.5) before adding substrates.

RESULTS

EFFECT OF CHANGING BUFFER PH AND Ca^{2+} ON MITOCHONDRIAL RELEASE OF H_2O_2 AND VOLUME AFTER INHIBITING COMPLEX I IN PYRUVATE-ENERGIZED MITOCHONDRIA

We first evaluated H_2O_2 release rates resulting from ROT-inhibited complex I in pyruvate-energized mitochondria at pHs 7.15, 6.9, and 6.5, each with H_2O (control) or with added 150 μM $CaCl_2$ (**Figures 1A–C, 2**). In the absence of $CaCl_2$, adding ROT caused a modest increase in H_2O_2 release rate at each pH. In the

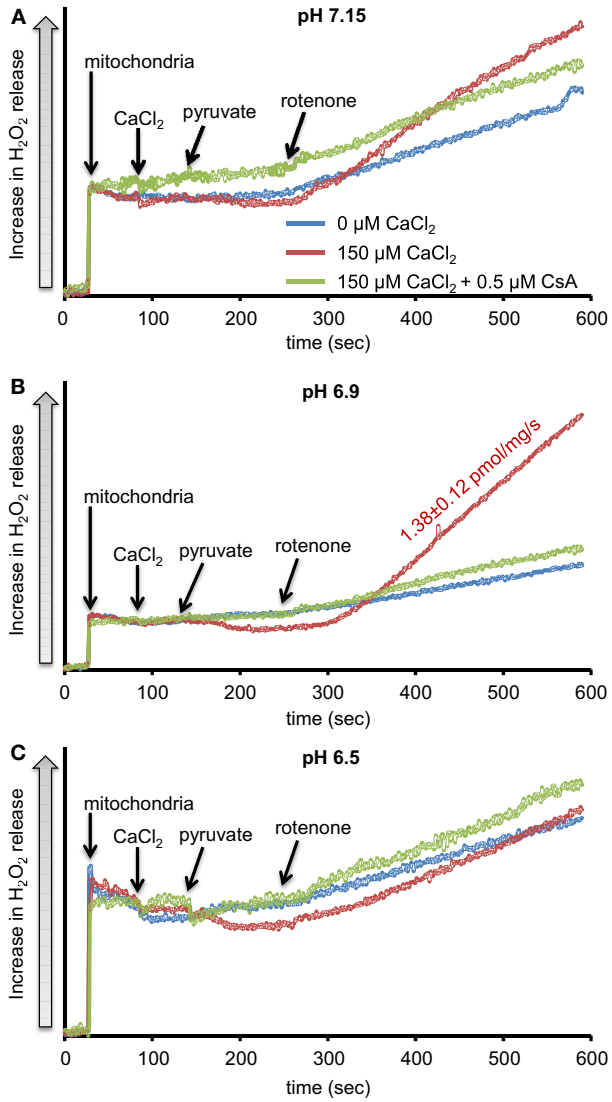

FIGURE 1 | Time-dependent changes in H_2O_2 release rates in isolated pyruvate-energized mitochondria after inhibiting complex I with rotenone (ROT) at a different pH: (A) pH 7.15, (B) pH 6.9, (C) pH 6.5. Mitochondria were added to buffer at 30 s, $CaCl_2$ at 90 s, pyruvate at 150 s, and ROT at 270 s. The blue trace represents no added $CaCl_2$, the red trace represents added $CaCl_2$ to 150 μM in the presence of 40 μM EGTA (≈ 220 nmol/mg free Ca^{2+}), and the green trace represents added $CaCl_2$ to 150 μM with cyclosporine A (CsA). H_2O_2 release was assessed using amplex red with horseradish peroxidase. Numbers indicate mean values ± SEM of pmol H_2O_2 generated/mg/s. $N = 4$ each.

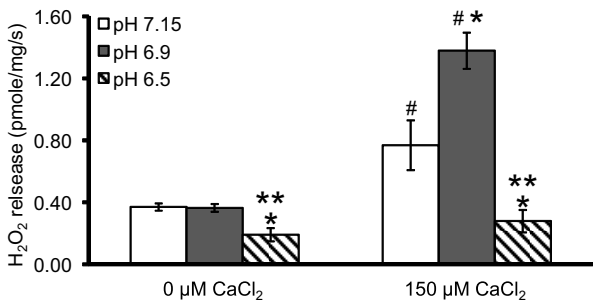

FIGURE 2 | Summary of the effects of pH and added $CaCl_2$ on H_2O_2 release rates in pyruvate-energized mitochondria after inhibiting complex I with ROT. Columns represent mean values ± SEM of pmol H_2O_2 emission/mg/s. $P < 0.05$ *Significant difference in H_2O_2 release rate at pH 6.9 or pH 6.5 vs. pH 7.15 within the same $CaCl_2$ group. **Significant difference in H_2O_2 release rate at pH 6.5 vs. pH 6.9 within the same $CaCl_2$ group. #Significant difference in H_2O_2 release rate in 150 μM $CaCl_2$ vs. 0 μM $CaCl_2$ for each pH group. $N = 4$ each.

presence of 150 μM $CaCl_2$, adding ROT caused a marked increase in H_2O_2 release rate at pH 6.9 (1.38 ± 0.12 pmol/mg/s) compared to pH 7.15 (0.37 ± 0.02 pmol/mg/s) or to pH 6.5 (0.28 ± 0.07 pmol/mg/s). Since high Ca^{2+} is known to induce mPTP opening (Camara et al., 2010), we tested the hypothesis that the Ca^{2+}-induced H_2O_2 increase is a result of mPTP opening. Addition of cyclosporine A (CsA; 0.5 μM; Sigma) prevented the large increase in H_2O_2 release, which was more apparent at pH 6.9 (0.39 ± 0.04 pmol/mg/s). To further evaluate the role of mPTP, we measured the corresponding mitochondrial volume changes in pyruvate-energized mitochondria in the same combinations of buffer pH and $CaCl_2$ with later addition of ROT (**Figures 3A–C**). Adding $CaCl_2$ alone did not significantly alter mitochondrial volume before addition of pyruvate. However, at pH 6.9 (**Figure 3B**) and 6.5 (**Figure 3C**), mitochondrial volume significantly increased with added 150 μM $CaCl_2$ after adding pyruvate, but volume was not significantly affected at pH 7.15 (**Figure 3A**). Additionally, mitochondrial volume did not change significantly in experiments without added $CaCl_2$. Adding CsA prevented the large increases in mitochondrial volume; adding ROT stopped any increases in volume (**Figures 3A–C**). The effect of adding superoxide SOD on H_2O_2 release under these conditions is given in Supplementary Materials.

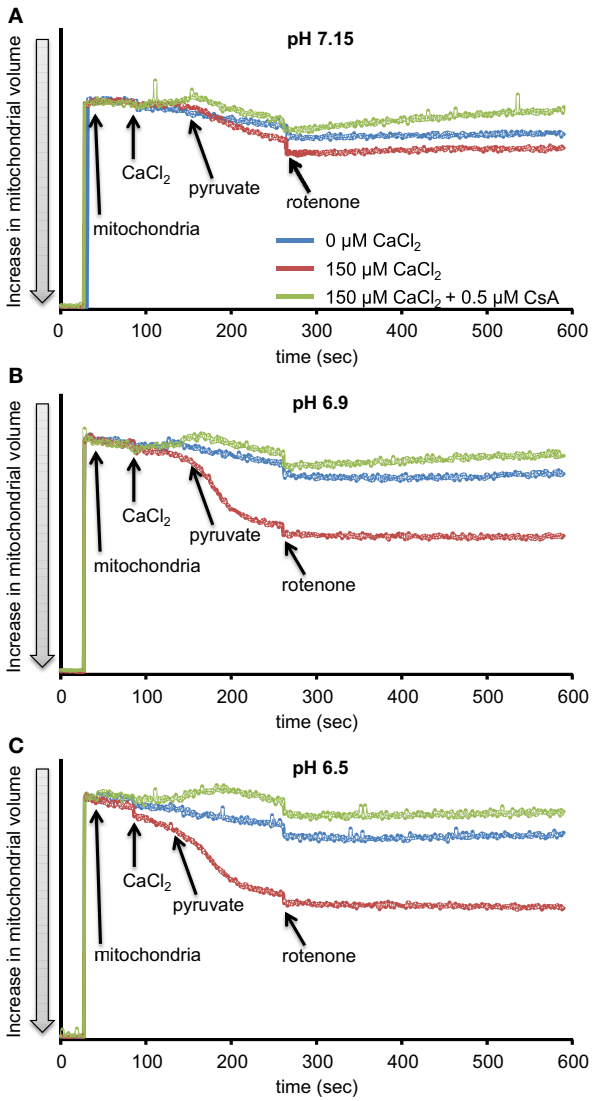

FIGURE 3 | Time-dependent changes in mitochondrial volume in isolated pyruvate-energized mitochondria after inhibiting complex I with ROT at a different pH: (A) pH 7.15, (B) pH 6.9, (C) pH 6.5. Mitochondria were added to buffer at 30 s, $CaCl_2$ at 90 s, pyruvate at 150 s, and ROT at 270 s. The blue trace represents no added $CaCl_2$, the red trace represents added $CaCl_2$ to 150 μM in the presence of 40 μM EGTA (≈ 220 nmol/mg free Ca^{2+}), and the green trace represents added $CaCl_2$ to 150 μM with cyclosporine A (CsA). Mitochondrial volume was assessed by measuring changes in 90° light scattering. $N = 3$ each.

EFFECT OF CHANGING BUFFER PH AND Ca^{2+} ON MITOCHONDRIAL RELEASE OF H_2O_2 AND VOLUME AFTER INHIBITING COMPLEX III IN SUCCINATE-ENERGIZED MITOCHONDRIA

We evaluated H_2O_2 release rates resulting from adding AA to succinate-energized mitochondria at pH 7.15, 6.9, or 6.5, without (control) or with added 150 μM $CaCl_2$ (**Figures 4A–C**, **5**). In the absence of $CaCl_2$ at all pHs tested, adding succinate prior to adding AA caused an increase in H_2O_2 release rate while later addition of AA reduced succinate-induced H_2O_2 release rate (**Figures 4A–C**). In the presence of 150 μM $CaCl_2$ without AA, adding succinate did not significantly increase H_2O_2 release. However, later addition of AA with added $CaCl_2$ (150 μM) caused a large increase in H_2O_2 release rate at all pHs with pH 7.15 showing the highest rate (35.2 ± 1.0 pmol/mg/s), followed by pH 6.9 (32.6 ± 0.8 pmol/mg/s), and then by pH 6.5 (23.7 ± 1.6 pmol/mg/s) (**Figures 4A–C**, **5**). Adding CsA prevented the Ca^{2+}-induced increase in H_2O_2 release rate resulting from AA treatment at each pH with the least effect on mPTP at pH 7.15. Because enzyme activity is pH dependent, we examined the effect of adding SOD to the buffer under the same experimental conditions. The effect of adding SOD on H_2O_2 release was minimal (Figures S.1, S.2). In parallel, we measured the corresponding mitochondrial volume changes in succinate-energized mitochondria in the same combinations of pH and extra-matrix $CaCl_2$ with later addition of AA (**Figures 6A–C**). Adding $CaCl_2$ alone did not

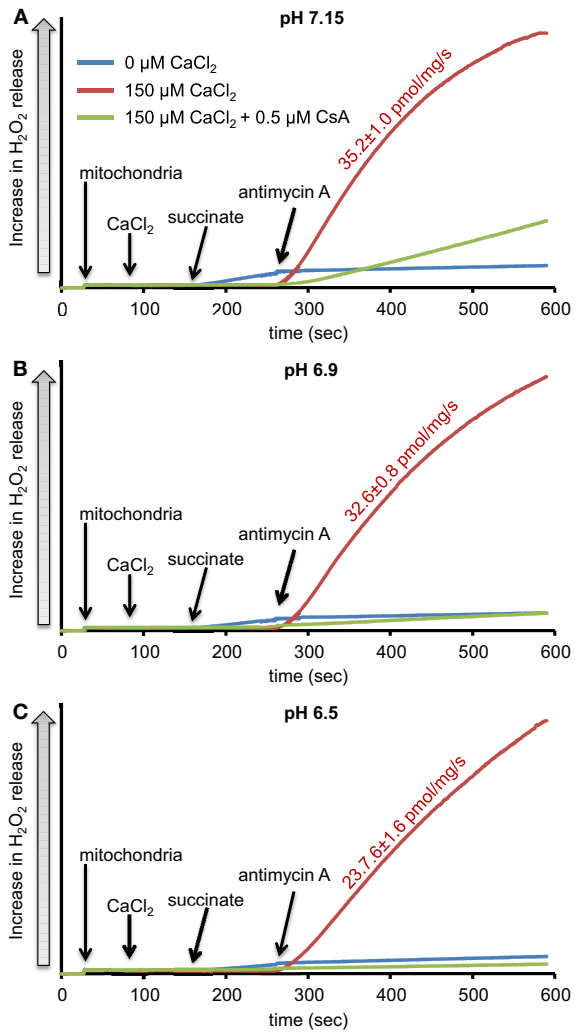

FIGURE 4 | Time-dependent changes in H_2O_2 release rates in isolated succinate-energized mitochondria after inhibiting complex III with antimycin A (AA) at a different pH: (A) pH 7.15, (B) pH 6.9, (C) pH 6.5. Mitochondria were added to buffer at 30 s, $CaCl_2$ at 90 s, succinate at 150 s, and AA at 270 s. The blue trace represents no added $CaCl_2$, the red trace represents added $CaCl_2$ to 150 μM in the presence of 40 μM EGTA (≈ 220 nmol/mg free Ca^{2+}), and the green trace represents added $CaCl_2$ to 150 μM with CsA. Numbers indicate mean values ± SEM of pmol H_2O_2 generated/mg/s. $N = 4$ each.

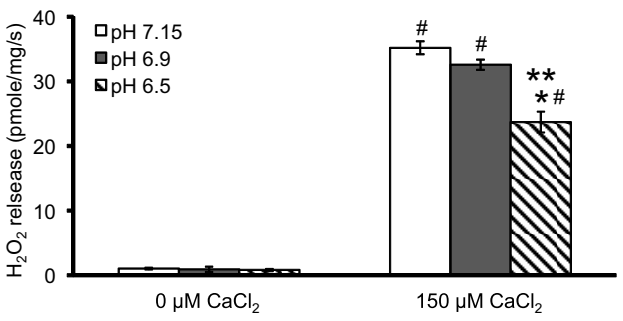

FIGURE 5 | Summary of the effects of pH and added $CaCl_2$ on rates of H_2O_2 release rate in succinate-energized mitochondria after inhibiting complex III with AA. Columns represent mean values ± SEM of pmol H_2O_2 generated/mg/s. $P < 0.05$ *Significant difference in H_2O_2 release rate at pH 6.9 or pH 6.5 vs. pH 7.15 within the same $CaCl_2$ group. **Significant difference in H_2O_2 release rate at pH 6.5 vs. pH 6.9 within the same $CaCl_2$ group. #Significant difference in H_2O_2 release rate in 150 μM $CaCl_2$ vs. 0 μM $CaCl_2$ for each pH group. $N = 4$ each.

significantly alter mitochondrial volume before adding succinate (**Figures 6A–C**). Later addition of succinate induced a significant increase in mitochondrial volume at all pHs which was prevented by CsA. Adding AA led to an attenuation of mitochondrial volume (**Figures 6A–C**).

EFFECT OF CHANGING BUFFER pH AND Ca^{2+} ON MITOCHONDRIAL RESPIRATION IN PYRUVATE AND SUCCINATE-ENERGIZED MITOCHONDRIA

Because $O_2^{\bullet -}$ generation is dependent on electron flux through the ETC and is a product of electron leak at several complexes, we evaluated the effects of substrate, pH, and $[Ca^{2+}]_e$ on mitochondrial respiration during states 2, 3, and 4 respiration (all of the above experiments were conducted during state 2). In pyruvate-energized mitochondria (**Figure 7A**), and before adding $CaCl_2$, there was no difference in state 2 respiration among all pH groups. Adding 150 μM $CaCl_2$ led to an increase in state 2 in the pH 7.15 group only. In succinate-energized mitochondria (**Figure 7B**), and before adding $CaCl_2$, there was no difference in state 2 respiration among all pH groups. However, adding 150 μM $CaCl_2$ led to similar decreases in state 2 respiration for all pH groups. States 3 and 4 respiration rates and the respective RCI values for each pH group without (control) or with addition of 150 μM $CaCl_2$ are summarized (**Table 1**). Adding 150 μM $CaCl_2$ decreased states 3 and 4 respiration and RCI under each of the two substrate and three pH conditions, except at pH 7.15 for state 4 respiration with pyruvate where state 4 respiration increased slightly but significantly. State 3 respiration and RCI were unaffected by pH except in two conditions: (1) pyruvate and 0 μM $CaCl_2$, in which they were reduced at pH 6.5, and (2) pyruvate and 150 μM $CaCl_2$, in which they were reduced at pH 6.9 and 6.5. State 4 respiration was unaffected by pH except in the condition pyruvate and 150 μM $CaCl_2$, in which it was reduced at pH 6.9 and 6.5.

DISCUSSION

The main goal of this work was to simulate in isolated heart mitochondria prevailing effects that can occur in cardiac IR injury such as excess Ca^{2+}, low pH, and impaired electron transfer at ETC complexes I and III, and to determine their impact on release of H_2O_2. In addition, we used either the complex I substrate pyruvate or the complex II substrate succinate to mimic substrate conditions that may prevail during IR. The first protocol (e.g., **Figure 3**) was intended to represent less severe IR injury (pyruvate as substrate, inhibited complex I > complex III, low Ca^{2+} loading, pH 7.15, 6.9); the second protocol was intended to represent more severe IR injury (succinate as substrate, inhibited complex III + complex I, high Ca^{2+} loading, pH 6.5). Under these latter conditions, H_2O_2 release rate was about 10 times higher, and the lower pH with either substrate condition

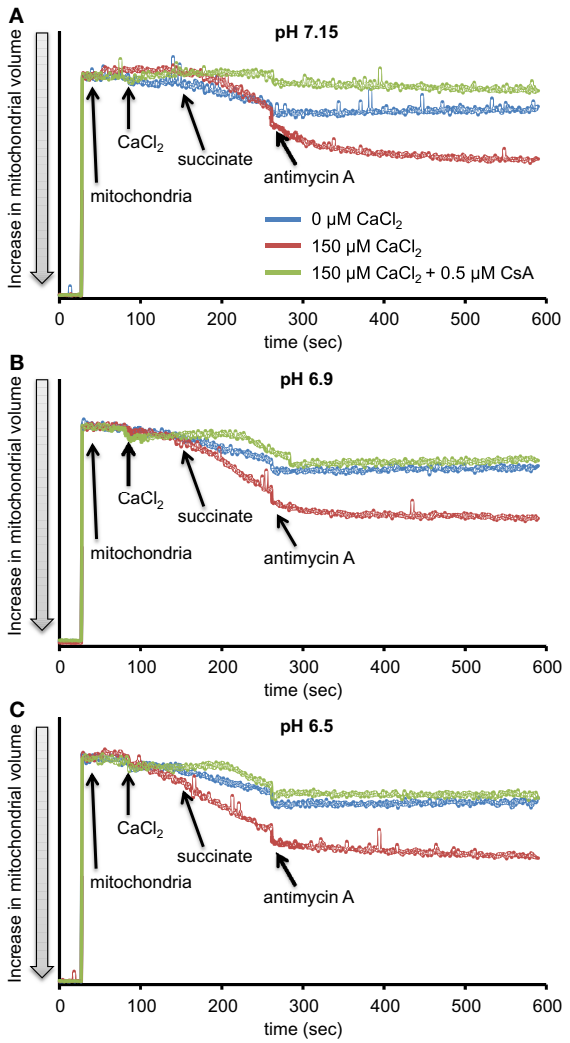

FIGURE 6 | Time-dependent changes in mitochondrial volume in isolated succinate-energized mitochondria after inhibiting complex III with AA at a different pH: (A) pH 7.15, (B) pH 6.9, (C) pH 6.5. Mitochondria were added to buffer at 30 s, $CaCl_2$ at 90 s, succinate at 150 s, and AA at 270 s. The blue trace represents no added $CaCl_2$, the red trace represents added $CaCl_2$to 150 μM in the presence of 40 μM EGTA (≈ 220 nmol/mg free Ca^{2+}), and the green trace represents added $CaCl_2$ with CsA. $N = 3$ each.

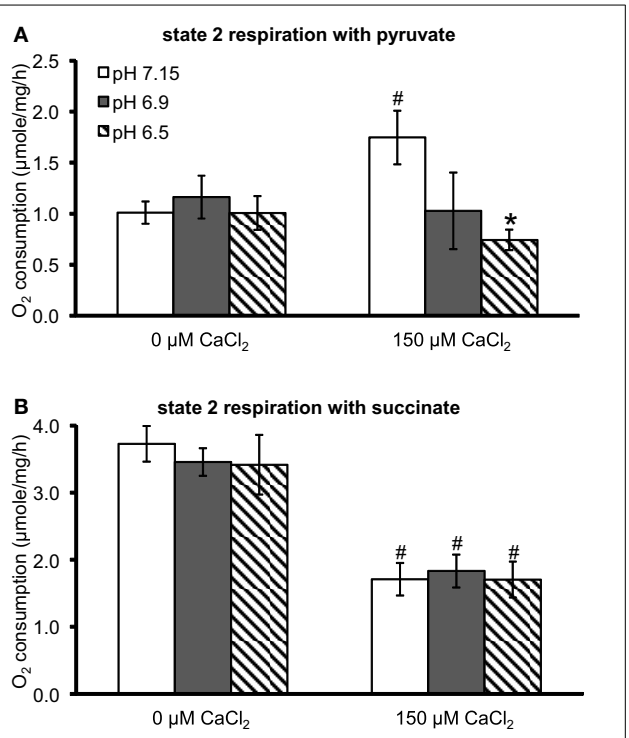

FIGURE 7 | Summary of the effects of pH and added $CaCl_2$ on O_2 consumption (μmol/mg/h) during state 2 respiration in (A) pyruvate-energized and (B) succinate-energized mitochondria. $P < 0.05$ *Significant difference in O_2 consumption at pH 6.9 or pH 6.5 vs. pH 7.15 within the same $CaCl_2$ group. #Significant difference in O_2 consumption in 150 μM $CaCl_2$ vs. 0 μM $CaCl_2$ for each pH group. O_2 consumption was measured in a respirometer. $N = 4$ each.

attenuated H_2O_2 release. Overall, our results show that in succinate energized mitochondria, increased buffer Ca^{2+} enhances mitochondrial H_2O_2 release rates resulting from complex I and III (AA) similarly at each pH, whereas in pyruvate energized mitochondria, a significant increase in H_2O_2 release rate resulting from complex I (ROT) occurs only at pH 6.9.

COMPLEX I AND COMPLEX III ARE PRIMARY SOURCES OF $O_2^{\bullet-}$ DURING ISCHEMIA AND REPERFUSION

It is well-documented that reperfusion after ischemia is associated with considerable ROS emission (Vanden Hoek et al., 1997; Becker et al., 1999; Kevin et al., 2003). It appears paradoxical that ROS are also produced during ischemia; but total mitochondrial anoxia is unlikely to exist even with extensive cardiac ischemia (Becker, 2004). It has been reported that isolated mitochondria produce H_2O_2 when O_2 is as low as 0.5 mM (pO_2 of about 10 mmHg) (Saborido et al., 2005). Mitochondrial pO_2 *in vivo* is only about 1–5 mmHg with normoxia, so mitochondria normally thrive in a low O_2 environment. The emission of ROS is due to excessive $O_2^{\bullet-}$ generation (likely during early mild ischemia when the redox potential is high) and to diminished ROS scavenging (likely during later severe ischemia when the redox potential is low).

In previous studies of IR injury in guinea pig isolated hearts, we observed a modest increase in $O_2^{\bullet-}$ generation during early ischemia (10–15 min) followed by a larger increase in $O_2^{\bullet-}$ generation during late ischemia (20–30 min) and a surge during early reperfusion (Kevin et al., 2003; Riess et al., 2004; Aldakkak et al., 2008a,b, 2011). Recently, we demonstrated in isolated mitochondria, under conditions simulating ischemia, that $CaCl_2$ addition in the presence of succinate resulted in enhanced H_2O_2 release when complex III was blocked (Aldakkak et al., 2013).

It is widely recognized that both mitochondrial complexes I and III play a crucial role in producing ROS during cardiac IR injury, particularly with induction of mPTP. "Triggering" amounts of ROS can be generated in isolated cardiomyocytes by photoactivation of tetramethylrhodamine derivatives that leads to membrane depolarization (mPTP induction) and a burst of ROS,

Table 1 | Effects of increasing concentrations of $CaCl_2$ on states 3, and 4, and respiratory control index (RCI, state 3/state 4) under different substrate and pH conditions.

	Substrate	μM $CaCl_2$	pH 7.15	pH 6.9	pH 6.5
State 3	Pyruvate	0	13.7 ± 0.8	14.6 ± 0.9	9.2 ± 1.3*
		150	9.0 ± 1.0‡	0.9 ± 0.4*‡	0.5 ± 0.1*‡
State 3	Succinate	0	11.4 ± 1.1	12.5 ± 0.5	9.9 ± 3.9
		150	2.0 ± 0.2‡	1.9 ± 0.3‡	1.9 ± 0.3‡
State 4	Pyruvate	0	1.1 ± 0.1	1.2 ± 0.1	1.3 ± 0.2
		150	2.3 ± 0.2‡	0.7 ± 0.3*‡	0.6 ± 0.2*‡
State 4	Succinate	0	3.9 ± 0.4	3.7 ± 0.3	3.2 ± 0.5
		150	2.4 ± 0.3‡	2.3 ± 0.3‡	2.1 ± 0.3‡
RCI (state 3/state 4)	Pyruvate	0	12.6 ± 0.7	12.0 ± 0.4	7.2 ± 0.6*§
		150	4.0 ± 1.1‡	1.3 ± 0.3*‡	1.0 ± 0.1*‡
RCI (state 3/state 4)	Succinate	0	3.1 ± 0.5	3.8 ± 0.4	3.5 ± 0.6
		150	0.9 ± 0‡	0.9 ± 0‡	0.9 ± 0.0‡

*$P < 0.05$ *Significant difference in states 3 and 4 and RCI in pH 6.9 or pH 6.5 vs. pH 7.15 within the same $CaCl_2$ group. §Significant difference in RCI in pH 6.5 vs. pH 6.9 within the same $CaCl_2$ group. ‡Significant difference in states 3 and 4 and RCI in 150 μM $CaCl_2$ vs. 0 μM $CaCl_2$ for each pH group. Note the marked effect of 150 μM $CaCl_2$ to depress RCI at each pH and the smaller effect of pH 6.5 with pyruvate and no $CaCl_2$ to reduce RCI.*

which is coined "ROS-induced ROS release (RIRR)" (Zorov et al., 2000). However, inducing mPTP opening with excess $CaCl_2$ in isolated liver mitochondria did not increase H_2O_2 release unless exogenous NADH was added to the buffer (Batandier et al., 2004). Because mPTP opening resulted in a ROT-sensitive impairment of complex I activity, they concluded that mPTP opening is associated with defective electron transfer within complex I, leading to $O_2^{\bullet-}$ release at that site. We did not add NADH to the buffer but we observed an increase in H_2O_2 release under either substrate condition when complex I and III inhibitors were used to mimic some conditions of IR injury. Despite these advances, the prevailing metabolic conditions during mild vs. severe IR injury that promote differential dysfunction of the complexes to cause excess $O_2^{\bullet-}$ generation and ROS emission in a vicious cycle of RIRR remain unclear.

In ischemia, decreasing O_2 levels and concomitant activation of glycolysis caused a decrease in pH, increased extra-matrix and matrix [Ca^{2+}], and eventually damage to complexes I and III due to oxidative stress (Rouslin, 1983). $O_2^{\bullet-}$ generated under these conditions is derived in part from complex I through forward electron transfer (FET) (Starkov et al., 2004). In the study above (Rouslin, 1983), it was reported that the activity of complex I decreased markedly after 20 min ischemia and that this decrease closely paralleled the decrease in mitochondrial O_2 uptake with NADH-linked substrates; it was also reported that the activity of complex III decreased at a more gradual rate during ischemia and that its rate of decrease paralleled that of succinate-supported O_2 uptake. As ischemia progresses, pH drops further and extra-matrix and matrix Ca^{2+} levels rise, while substrate utilization switches from primarily pyruvate to mostly succinate, which accumulates from 0.2 to 0.4 mM during normoxia to 4–7 mM during ischemia or hypoxia (Kakinuma et al., 1994; Starkov et al., 2004). Based on these studies, we used pyruvate + ROT to approximate the condition of impaired electron transfer via complex I to complex III, and succinate + AA to approximate the condition of enhanced utilization of succinate at complex II and impaired electron transfer through complex I (by RET) and III. Our protocols were also based on a summary of data (Lukyanova, 2013) stating that the switch from normoxia to hypoxia increased succinate utilization by complex II from about 25–35% to 65–85% while complex I activity was mostly inhibited. Under this condition, complex III appears to be damaged by ROS due to the initial $O_2^{\bullet-}$ generated at complex I, which is analogous to AA inhibition of complex III (Musatov and Robinson, 2012). Given these conditions and the diminished $\Delta\Psi_m$, ROS production during severe IR injury likely results from FET including complex II and III. Therefore, our experiments with succinate + AA may mimic the conditions of $O_2^{\bullet-}$ generated at both complex I and III during IR injury.

BIPHASIC EFFECTS OF pH ON mPTP LEADS TO VARYING H_2O_2 PRODUCTION FROM COMPLEX I

Adding $CaCl_2$ and lowering pH both modulated H_2O_2 production in pyruvate-energized mitochondria after adding ROT (**Figures 1A–C**, **2**). Based on previous studies showing a distinct early phase of H_2O_2 production during mild ischemia correlated with the timing of complex I damage, we hypothesized that pyruvate-energized mitochondria with inhibited complex I and added $CaCl_2$ would show an increased H_2O_2 release rate as buffer pH decreased. Indeed, a sharp rise in H_2O_2 production was observed at pH 6.9 on addition of $CaCl_2$, which was not seen at pH 7.15. However, as pH decreased further to 6.5, a rise in H_2O_2 did not occur. It is unclear what the exact mechanism is for the large increase in H_2O_2 release in pyruvate-supported mitochondria at pH 6.9 with added $CaCl_2$. It is important to note that under these conditions, there was a significant increase in mitochondrial volume, probably indicating mPTP opening. Indeed, addition of CsA to desensitize mPTP prevented the increase in mitochondrial volume and reduced H_2O_2 release to levels similar to those observed at pH 7.15. Nonetheless, mPTP opening occurred also at pH 6.5 with high $CaCl_2$ as indicated by the increase in mitochondrial volume that was prevented with CsA; but this was not associated with a large increase in H_2O_2 release.

mPTP opening can occur during mitochondrial Ca^{2+} overload (Orrenius et al., 2003), whereas a low mitochondrial pH is associated with a reduced probability of mPTP opening. For example, reoxygenation or reperfusion under acidic conditions is associated with much lower ROS emission (Haworth and Hunter, 1979; Halestrap, 1991; Bernardi et al., 1992). However, a previous study (Halestrap, 1991), with glutamate/malate-energized mitochondria isolated from rat hearts, showed a graded effect of pH on the probability of mPTP opening with the least likelihood of opening at pH 6.0 and below. At pHs above 6.0 there was a significant increase in the probability of mPTP opening. Indeed, at pH 6.5, a Ca^{2+} -induced increase in volume, presumably through mPTP opening, was demonstrated to be

greater than 40% of the increase seen at pH 7.4; and pH 6.9 had approximately 75% of the volume increase seen at pH 7.4 (Halestrap, 1991).

mPTP opening is proposed to increase ROS emission through three mechanisms: a loss of glutathione leading to decreased ROS scavenging, a loss of cytochrome *c* leading to increased reduction of upstream ETC complexes and subsequent electron loss and diminished scavenging, and an increase in ROS derived from the α-ketoglutarate dehydrogenase complex due to loss of NAD^+ (Camara et al., 2010, 2011; Toledo et al., 2014). In our study different probabilities of mPTP opening at pH 6.9 and 6.5 may be responsible for the disparity in H_2O_2 levels generated at these two pHs. Limited opening of mPTP at pH 6.5 may be responsible for H_2O_2 generation at levels not sufficient to induce RIRR, whereas at pH 6.9 the increased opening of mPTP may induce RIRR. Additionally, the lack of increased H_2O_2 generation and mitochondrial volume at pH 7.15, when compared to pH 6.9, might be related to an increase in inhibition of complex I with high $CaCl_2$ as pH increases (Sadek et al., 2004; Chen et al., 2010). The decrease in complex I activity leading to decreased ROS production might in turn prevent RIRR and subsequent opening of the mPTP (Zorov et al., 2006). Thus, the combined effects of inhibited complex I activity and mPTP opening at pH 6.9 might explain the elevation in H_2O_2 release rates observed at pH 6.9, but not at pHs 7.15 and 6.5.

pH-DEPENDENT $O_2^{\bullet-}$ GENERATION FROM COMPLEXES I AND III AND STABILITY OF SEMIQUINONE RADICAL

Adding $CaCl_2$ and altering pH also modulated H_2O_2 generation in succinate-energized mitochondria with added AA (**Figures 4A–C**). With AA, mitochondria in buffer with high $CaCl_2$ showed a dramatic rise in H_2O_2 release at all pHs. The increase in H_2O_2 corresponded to an increase in pH. In addition, an increase in mitochondrial volume occurred at each pH in the presence of high $CaCl_2$, and these conditions led to increased H_2O_2 release, suggesting a role for mPTP opening. Indeed, both the increases in H_2O_2 release and volume were inhibited by adding CsA at each pH. H_2O_2 generation under these conditions is likely caused by FET from complex II through complex III, because adding AA decreased $\Delta\Psi_m$, which would prevent RET from occurring. In this case, the primary source of H_2O_2 is that derived from $O_2^{\bullet-}$ generated at the Q_o site of complex III.

Mitochondrial pH may have a significant role in moderating $O_2^{\bullet-}$ generation by complex III. Matrix alkalinization (higher pH) tended to stabilize the semiquinone radical at the Q_o site (Selivanov et al., 2008). This was proposed to result from decreased binding of H^+ ions necessary to drive the Q cycle forward (Selivanov et al., 2008). Stability of the semiquinone radical leads to increased likelihood of direct transfer of an electron to an O_2 molecule, leading to the formation of $O_2^{\bullet-}$ (Selivanov et al., 2008). Additionally, because AA blocks the oxidation of semiquinone at Q_i and the transfer of an electron from the Q_o site, this can lead to increased $O_2^{\bullet-}$ generation from the Q_o site, which may be analogous to impaired complex III function during ischemia (Chen et al., 2008; Musatov and Robinson, 2012). Consequently, in our experiments, the increased H_2O_2 release rate at a high pH is possibly related to the increased stability of semiquinone leading to increased direct electron donation to O_2 to generate $O_2^{\bullet-}$.

Ca^{2+} -INDUCED mPTP OPENING AND MITOCHONDRIAL RESPIRATION

Mitochondrial uncouplers like dinitrophenol tend to increase respiration to counteract a decline in $\Delta\Psi_m$ due to H^+ leak. But others have reported that it is not unusual for mitochondrial uncouplers or uncoupling events such as mPTP opening to inhibit succinate-supported state 2 respiration. Mitochondrial uncouplers can retard succinate oxidation under some conditions (Papa et al., 1969). In the absence of ROT, and with succinate in high concentrations (conditions similar to those used in our study), mitochondrial uncouplers have been found to inhibit succinate oxidation due to the formation of oxaloacetate (Wojtczak et al., 1969; Vik and Hatefi, 1981; Kotlyar and Vinogradov, 1984; Drose, 2013).

In our study, the lower state 2 respiration after adding $CaCl_2$ with succinate (**Figure 7B**) at each pH, may be due to a greater collapse in $\Delta\Psi_m$ due to the excess influx of Ca^{2+}. In contrast, the higher state 2 respiration after adding $CaCl_2$ with pyruvate (**Figure 7A**) at pH 7.15 may be a result of enhanced H^+ pumping at complex I by this NADH-linked substrate; however, as the trans-membrane pH potential is increased (and thus the proton motive force), a faster respiration might not be needed to maintain $\Delta\Psi_m$. The RCI for pyruvate was reduced at pH 6.5 likely because of an uncoupling effect due to H^+ leak with slower ATP production (**Table 1**). The RCI for succinate, which is much lower than that for pyruvate, appears to stem from the much higher basal respiratory rate for succinate vs. pyruvate (**Figures 7B** vs. **7A**). In the absence of $CaCl_2$, mitochondria showed well-coupled oxidative phosphorylation with both substrates at each pH, except in pyruvate-energized mitochondria at pH 6.5, in which case the RCI was lower, indicating relatively less coupling. The effect of added $CaCl_2$ on states 3 and 4 respirations and RCI with either substrate or at any pH is likely a result of marked uncoupling due to mPTP opening (increased mitochondrial volume) because this was sensitive to CsA (**Figures 3**, **6**).

SUMMARY, CONCLUSION, AND PERSPECTIVE

In conclusion, in our previous studies using the isolated, beating heart model of 30 min global ischemia, we demonstrated two phases of increased $O_2^{\bullet-}$ generation, an early phase (10–20 min) that emits low to moderate $O_2^{\bullet-}$ levels and a late phase (20–30 min) that emits higher $O_2^{\bullet-}$ levels just before a surge in $O_2^{\bullet-}$ release at the beginning of reperfusion. The present study sheds novel insights into the modulatory effect of matrix pH in Ca^{2+}-induced mitochondrial H_2O_2 release. The early or mild phase of H_2O_2 release due to $O_2^{\bullet-}$ generation at complex I could be related to differential effects of pH on the mPTP, which allows H_2O_2 production at the pH observed during early or mild ischemia (pH 6.9) but not at the lower or higher pHs. The late or severe phase of H_2O_2 release due to $O_2^{\bullet-}$ generation primarily at complex III, but also at complex I, may also be dependent on mPTP opening, but H_2O_2 production is intensified with increasing pH. Therefore, it is possible that the surge in H_2O_2 production commonly observed on reperfusion results from $O_2^{\bullet-}$ generated from complex III as the pH rises gradually with mPTP opening.

Although both complex I and III contribute simultaneously to H_2O_2 production during IR, our results suggest that the role of each respiratory complex is not static but rather changes dynamically as the pH changes. Thus, each complex may play a more prominent role during a certain period of IR. Being cognizant of this information is important as it can be used to reduce ROS emission as ischemia progresses by targeting each complex separately, or possibly by manipulating the pH using Na^+/Ca^{2+} and/or Na^+/H^+ exchange inhibitors, e.g., by maintaining a more alkaline environment during early ischemia and a more acidic environment during late ischemia/early reperfusion, to reduce $O_2^{\bullet-}$ generation at complex I and III.

POTENTIAL IMITATIONS

Although we have attempted to simulate some of the conditions in mitochondria that may occur during authentic cardiac IR injury, there are several shortcomings to this approach: (1) Our experimental design did not allow us to mimic the timing of ROS production during ischemia, or during reperfusion after ischemia, or to allow for the possible redox conditions associated with varying ROS scavenging capacity during IR injury. (2) We completely blocked electron transfer sites using inhibitors; *in vivo* it is known that IR injury impairs electron transfer, but does not block it completely. (3) IR injury could impede electron transfer at other sites (e.g., myxothiazol prevents semiquinone formation at the Q_o site), which could inhibit $O_2^{\bullet-}$ generation at that site, but stimulate it at another site. (4) On the other hand, IR injury may cause $O_2^{\bullet-}$ generation at other sites not examined (e.g., the flavin site of complex I). (5) Free fatty acids are a normal substrate for mitochondria and they were absent in this study. (6) The use of succinate + AA cannot distinguish $O_2^{\bullet-}$ generation from complex I vs. III without knowledge of the redox state and $\Delta\Psi_m$ or the presence of ROT to prevent RET. (7) Changes in matrix pH and Ca^{2+} during IR injury may not arise solely due to changes in the cytosol, but rather in the matrix in response to bioenergetic dysfunction and possibly loss of mitochondrial buffering capacity. (8) It is very difficult to directly assess $O_2^{\bullet-}$ generation in mitochondria; thus a variation in the redox potential during IR injury can lead to differential dismutation of $O_2^{\bullet-}$ to H_2O_2, an additional factor not assessed in our simulated ischemia conditions.

AUTHOR CONTRIBUTIONS

DL Conducted the experiments, analyzed data, wrote the first draft. AC Assisted in developing the design of the study. Made critical revisions of the manuscript in development and after review. DS Assisted in developing the design of the study. Made critical revisions of the manuscript and Supplementary Materials in development and during revisions. RL Conducted supplemental experiments and assisted in suggestions to improve the revised manuscript and Supplementary Materials. MA Developed the structure, argument, and design of the study. Made critical revisions of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fphys.2015.00058/abstract

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Functional crosstalk between the mitochondrial PTP and K_{ATP} channels determine arrhythmic vulnerability to oxidative stress

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Background: Mitochondrial permeability transition pore (mPTP) opening is a terminal event leading to mitochondrial dysfunction and cell death under conditions of oxidative stress (OS). However, mPTP blockade with cyclosporine A (CsA) has shown variable efficacy in limiting post-ischemic dysfunction and arrhythmias. We hypothesized that strong feedback between energy dissipating (mPTP) and cardioprotective (mK_{ATP}) channels determine vulnerability to OS.

Methods and Results: Guinea pig hearts ($N = 61$) were challenged with H_2O_2 (200 μM) to elicit mitochondrial membrane potential ($\Delta\Psi_m$) depolarization. High-resolution optical mapping was used to measure $\Delta\Psi_m$ or action potentials (AP) across the intact heart. Hearts were treated with CsA (0.1 μM) under conditions that altered the activity of mK_{ATP} channels either directly or indirectly via its regulation by protein kinase C. mPTP blockade with CsA markedly blunted ($P < 0.01$) OS-induced $\Delta\Psi_m$ depolarization and delayed loss of LV pressure (LVP), but did not affect arrhythmia propensity. Surprisingly, prevention of mK_{ATP} activation with the chemical phosphatase BDM reversed the protective effect of CsA, paradoxically exacerbating OS-induced $\Delta\Psi_m$ depolarization and accelerating arrhythmia onset in CsA treated compared to untreated hearts ($P < 0.05$). To elucidate the putative molecular mechanisms, mPTP inhibition by CsA was tested during conditions of selective PKC inhibition or direct mK_{ATP} channel activation or blockade. Similar to BDM, the specific PKC inhibitor, CHE (10 μM) did not alter OS-induced $\Delta\Psi_m$ depolarization directly. However, it completely abrogated CsA-mediated protection against OS. Direct pharmacological blockade of mK_{ATP}, a mitochondrial target of PKC signaling, equally abolished the protective effect of CsA on $\Delta\Psi_m$ depolarization, whereas channel activation with 30 μM Diazoxide protected against $\Delta\Psi_m$ depolarization ($P < 0.0001$). Conditions that prevented mK_{ATP} activation either directly or indirectly via PKC inhibition led to accelerated $\Delta\Psi_m$ depolarization and early onset of VF in response to OS. Investigation of the electrophysiological substrate revealed accelerated APD shortening in response to OS in arrhythmia-prone hearts.

Conclusions: Cardioprotection by CsA requires mK_{ATP} channel activation through a PKC-dependent pathway. Increasing mK_{ATP} activity during CsA administration is required for limiting OS-induced electrical dysfunction.

Keywords: mitochondria, oxidative stress, permeability transition pore, mitochondrial K_{ATP} channel, arrhythmia

INTRODUCTION

Mitochondria are central mediators of the cardiac response to oxidative stress (OS), as they respond to reactive oxidative species (ROS) through a host of ROS sensitive channels, which can either amplify or limit ROS-induced injury (O'Rourke et al., 2007). Of key importance to OS-induced mitochondrial dysfunction are the inner membrane anion channel (IMAC) and components of the mitochondrial permeability transition pore (mPTP). Both channel complexes activate in response to rising ROS levels. However, as described by Aon and colleagues, they exhibit a hierarchal activation pattern (Aon et al., 2007): IMAC activates first in response to moderate levels of OS followed by the activation of the large conductance mPTP, which leads to irreversible mitochondrial membrane potential ($\Delta\Psi_m$) depolarization (i.e., induction of the mitochondrial permeability transition, MPT) (Aon et al., 2007). Indeed, both channels have been implicated in mitochondrial dysfunction through a regenerative, autocatalytic process known as ROS-induced ROS-release (RIRR) which can culminate in electrical dysfunction or cell death (Zorov et al., 2000, 2006; Yang et al., 2010; Biary et al., 2011; Akar, 2013).

While the role of the mPTP in the activation of necrotic cell death pathways is well established, we and others have

demonstrated the importance of IMAC in OS-induced arrhythmias (Akar et al., 2005; Akar and O'Rourke, 2011). In those studies, IMAC (but not mPTP) blockade effectively abrogated pathological OS-induced $\Delta\Psi_m$ and action potential (AP) oscillations and prevented post-ischemic arrhythmias (Akar et al., 2005). It is important to note, however, that our previous studies focused on relatively short episodes of ischemia-reperfusion (I/R) injury which did not result in myocardial infarction (MI) (Akar et al., 2005; Lyon et al., 2010). Given the hierarchal nature of mitochondrial channel activation (Aon et al., 2007), we hypothesized that the mPTP may only play a prominent role under conditions of more extreme OS. Indeed, the immunosuppressive agent, Cyclosporin A (CsA), a desensitizer of the mPTP in the heart through its effect on Cyclophilin-D (CyP-D), has been shown to be effective in reducing infarct size in patients (Piot et al., 2008; Hausenloy et al., 2012). Despite these encouraging clinical findings, the efficacy of CsA in preventing arrhythmias is unclear (Arteaga et al., 1992; Ko et al., 1997; Schreiner et al., 2004), and recent experimental, preclinical (Lie et al., 2008), and clinical findings (Ghaffari et al., 2013) have cast new doubts regarding the overall utility and safety profile of CsA.

Mitochondria play a dual role: on the one hand, they initiate cell death and injury pathways through energy dissipating channels, such as the mPTP, but on the other, they act as central mediators of cardioprotection (Penna et al., 2013). Indeed, multiple stimuli (i.e., ischemic pre- and post-conditioning protocols, pharmacological agents and volatile anesthetics) limit cardiac damage by activating powerful cardioprotective signaling cascades which converge on mitochondria, in large part, through mitochondrial ATP-sensitive K (mK_{ATP}) channels (Liu et al., 1998, 1999; Sato and Marban, 2000; Garlid et al., 2009). Whether mK_{ATP} channels functionally interact with components of the mPTP in a manner that modulates the response of the heart to OS is unclear. In the present study, we set out to address this issue directly in a model of acute OS that was specifically designed to elicit significant $\Delta\Psi_m$ depolarization and electrical dysfunction. We found that the efficacy of CsA in limiting OS-induced mitochondrial and electrical dysfunction was dictated by strong functional crosstalk between the mPTP and mK_{ATP} channels through a protein kinase C (PKC)-dependent pathway. Our findings highlight the importance of enhancing mK_{ATP} channel activity during CsA administration for limiting OS-induced electrical dysfunction, and may explain discrepant reports of the utility and potential toxicity of CsA.

MATERIALS AND METHODS

All procedures involving the handling of animals were approved by the Animal Care and Use Committee of the Mount Sinai School of Medicine and adhered with the Guide for the Care and Use of Laboratory Animals published by the *National Institutes of Health*. Guinea pig hearts ($N = 61$) were rapidly excised, washed with ice cold cardioplegic solution, transferred to a Langendorff apparatus, and retrogradely perfused through the aorta with oxygenized (95% O_2–5% CO_2) Tyrodes solution containing (in mM): 130 NaCl, 1.2 $MgSO_4$, 25 $NaHCO_3$, 4.75 KCl, 5 Dextrose, and 1.25 $CaCl_2$ at 36 ± 1°C. Perfusion pressure was maintained at 60–65 mmHg by adjusting perfusion flow rate. Hearts were suspended in the buffer filled, temperature controlled chamber, as we have recently reported (Jin et al., 2010; Lyon et al., 2010). Volume-conducted electrocardiograms were recorded for rhythm analysis using non-contact silver electrodes placed within the chamber. ECG signals were recorded continuously throughout the entire *ex vivo* perfusion protocol. Left ventricular (LV) cavity pressure (LVP) was measured using a buffer filled latex balloon (Harvard apparatus) that was carefully inserted through the mitral valve into the LV cavity. Signals were amplified (ECG100-MP150 Amplifier, Biopac Systems, CA, USA) and displayed in real-time using the *AcqKnowledge 3.9* software package (Biopac Systems). Hearts were positioned such that the mapping field was centered over a $4 \times 4\text{-mm}^2$ region of LV epicardium, midway between apex and base. These preparations remain stable for over 4 h of perfusion.

HIGH-RESOLUTION OPTICAL $\Delta\Psi_m$ IMAGING IN *EX VIVO* PERFUSED GUINEA PIG HEARTS

We used a validated semi-quantitative imaging technique of optical $\Delta\Psi_m$ mapping using the $\Delta\Psi_m$-sensitive dye tetramethylrhodamine methylester (TMRM) (Jin et al., 2010; Lyon et al., 2010; Smeele et al., 2011; Nederlof et al., 2013). This method allows the assessment of mitochondrial function at a subcellular resolution within the intact organ (Jin et al., 2010; Lyon et al., 2010). Briefly, following cannulation, hearts were allowed to stabilize for 20 min at physiological temperature. Hearts were then stained with TMRM (250 nM; Molecular Probes Inc.) mixed in a 500 mL volume of Tyrodes solution (dye loading phase) for 20 min. This was followed by a 20–30 min dye washout phase. TMRM background fluorescence intensity was measured periodically (in 1 min intervals) throughout the entire experiment using a 6400 pixel CCD based optical imaging approach that allowed the measurement of normalized $\Delta\Psi_m$ with subcellular resolution (50 μm) over a $4 \times 4\text{-mm}^2$ window of the epicardial surface. To measure TMRM background fluorescence, hearts were excited with filtered light (525 ± 20 nm) emitted from a quartz tungsten halogen lamp (Newport Corporation, CT, USA). Emitted fluorescence was filtered (585 ± 20 nm for TMRM) and focused onto a high-resolution CCD camera (Scimeasure, GA, USA). During dye washout, the stability of TMRM background fluorescence was evaluated in real-time, as this baseline level served for normalization purposes during OS. In all experiments, the dye washout phase was associated with stable signal intensity.

High-throughput analysis of optical signals was performed using custom designed software. Peak emitted TMRM fluorescence signal from each of 6400 pixels was measured before and after excitation. TMRM background fluorescence was baseline corrected by subtracting fluorescence levels before dye staining (<0.1%) for each pixel. Background corrected TMRM fluorescence ($\Delta\Psi_m$) during the OS protocol was then normalized to the value of steady-state TMRM fluorescence achieved during the dye washout phase for each of the 6400 individual pixels. Normalized $\Delta\Psi_m$ measurements during OS across the imaged $4 \times 4\text{-mm}^2$ region of the heart were plotted as contour maps using *Delta Graph 5.6* (Red Rock Software).

HIGH-RESOLUTION OPTICAL ACTION POTENTIAL MAPPING IN *EX VIVO* PERFUSED HEARTS

For optical AP mapping studies, hearts were stained with di-4-ANEPPS for 10 min as previously described (Akar et al., 2005; Xie et al., 2013). Hearts were paced at a steady-state pacing cycle length (PCL) of 300 ms. Unlike $\Delta\Psi_m$ imaging, optical AP mapping requires motion suppression; hence 10 mM BDM was used in this subset of studies.

EX VIVO MODELS OF ACUTE OS LEADING TO IRREVERSIBLE $\Delta\Psi_m$ DEPOLARIZATION

Perfusion of hearts with H_2O_2 is a well-established model of acute OS that results in triggered activity (Sato et al., 2009) as well as sustained atrial (Morita et al., 2010) and ventricular tachyarrhythmias (Morita et al., 2009; Biary et al., 2011). Following dye washout and stabilization, hearts were perfused with 200 μM H_2O_2 (Sigma-Aldrich) in Tyrodes for 30 min to elicit OS. We found that this model consistently gives rise to the regenerative process of RIRR (Biary et al., 2011), which culminates in significant $\Delta\Psi_m$ depolarization, contractile and electrical dysfunction (**Figure 1**). This model served as the platform for investigating the role of mitochondrial ion channel complexes in the modulation of OS-induced $\Delta\Psi_m$ and arrhythmias. Specifically, we focused on the role of the mPTP and its crosstalk with the mK$_{ATP}$. The following agents and concentrations were used in the present study: (a) CsA (0.1 μM, mPTP blocker), (b) Chelerythrine Chloride (CHE, 10 μM, PKC inhibitor), (c) 5-Hydroxydecanoate (5-HD, 100 μM, mK$_{ATP}$ blocker), and (d) diazoxide (DZX, 30μM, mK$_{ATP}$ agonist). Drug delivery was initiated 10 min before OS and was maintained throughout the entire protocol.

STATISTICAL ANALYSIS

Values were expressed as mean ± SE. Differences between two groups were compared using the Student's *t*-test and were considered significant for $p < 0.05$.

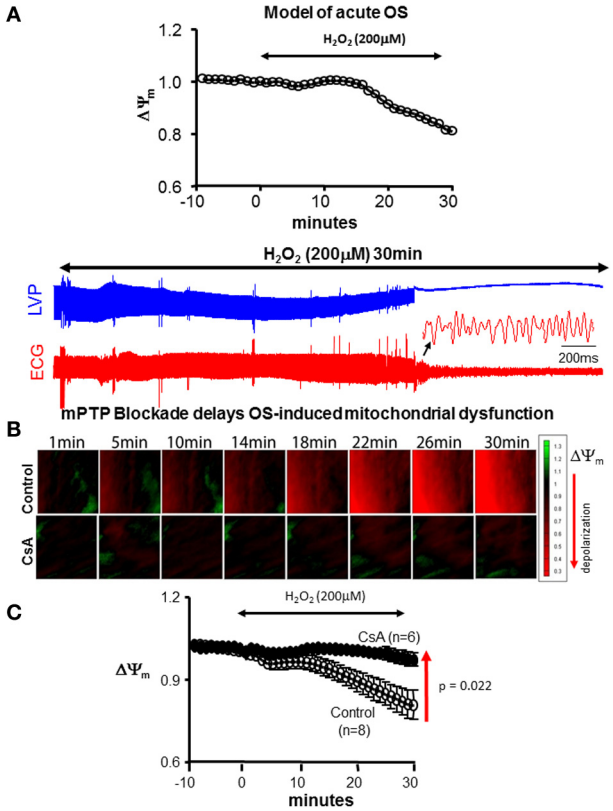

FIGURE 1 | CsA delays OS-induced mitochondrial and contractile dysfunction. (A) Model of acute OS. Continuous 200 μM H_2O_2 perfusion for 30 min leads to $\Delta\Psi_m$ depolarization and initiation of VF. **(B)** Sequences of isopotential contour maps depicting the spatio-temporal distribution of $\Delta\Psi_m$ in representative control (untreated) and CsA-treated hearts following challenge with H_2O_2 (200 μM) for 30 min. **(C)** Average $\Delta\Psi_m$ response to acute OS in all control and CsA treated hearts.

RESULTS

ACUTE MODEL OF OS-INDUCED MITOCHONDRIAL AND ELECTRICAL DYSFUNCTION

The main objective of the present work was to test the efficacy of mPTP blockade in protecting against OS-induced mitochondrial and electrical dysfunction. To that end, we used a simple *ex vivo* model of acute OS by H_2O_2 challenge (**Figure 1A**). This consistently resulted in significant $\Delta\Psi_m$ depolarization, contractile, and electrical dysfunction. Within 40 min of H_2O_2, 5/6 hearts underwent spontaneous onset of VF with the remaining heart exhibiting electrical silence. As such, this model served as a reliable platform for investigating the role of mitochondrial ion channel complexes in the functional modulation of OS-induced mitochondrial and electrical dysfunction.

CsA PROTECTS AGAINST OS-INDUCED MITOCHONDRIAL AND CONTRACTILE BUT NOT ELECTRICAL DYSFUNCTION

We began by investigating the efficacy of CsA in altering the functional response of hearts to acute OS. Shown in **Figure 1B** are $\Delta\Psi_m$ isopotential contour maps from untreated (control) and CsA-treated hearts following challenge with H_2O_2. Also shown are the average normalized $\Delta\Psi_m$ responses from all hearts. As expected, H_2O_2 challenge resulted in significant $\Delta\Psi_m$ depolarization in control hearts. On average, $\Delta\Psi_m$ was reduced by 22.1% within 30 min of H_2O_2 perfusion. Remarkably, CsA treatment completely abolished this response, as $\Delta\Psi_m$ remained fully polarized during the same time-course in CsA-treated compared to untreated control hearts (**Figure 1**). Following 30 min of H_2O_2 challenge, $\Delta\Psi_m$ was 19.3% greater ($p = 0.021$) in CsA-treated compared to control hearts.

We next tested whether modulation of OS-induced $\Delta\Psi_m$ depolarization and its prevention by CsA had a functional impact in terms of contractile (**Figures 2A–C**) and electrical (**Figures 2D,E**) properties. As expected, H_2O_2 perfusion in control hearts resulted in a gradual decrease and eventual loss of contractile function. Interestingly, prevention of $\Delta\Psi_m$ depolarization by CsA was associated with relative protection against contractile dysfunction as the loss of LVP was delayed by >8 min, $p = 0.01$ (**Figure 2**). We next investigated whether protection against OS-induced mitochondrial dysfunction by CsA translated into an electrical benefit by either preventing or delaying the onset

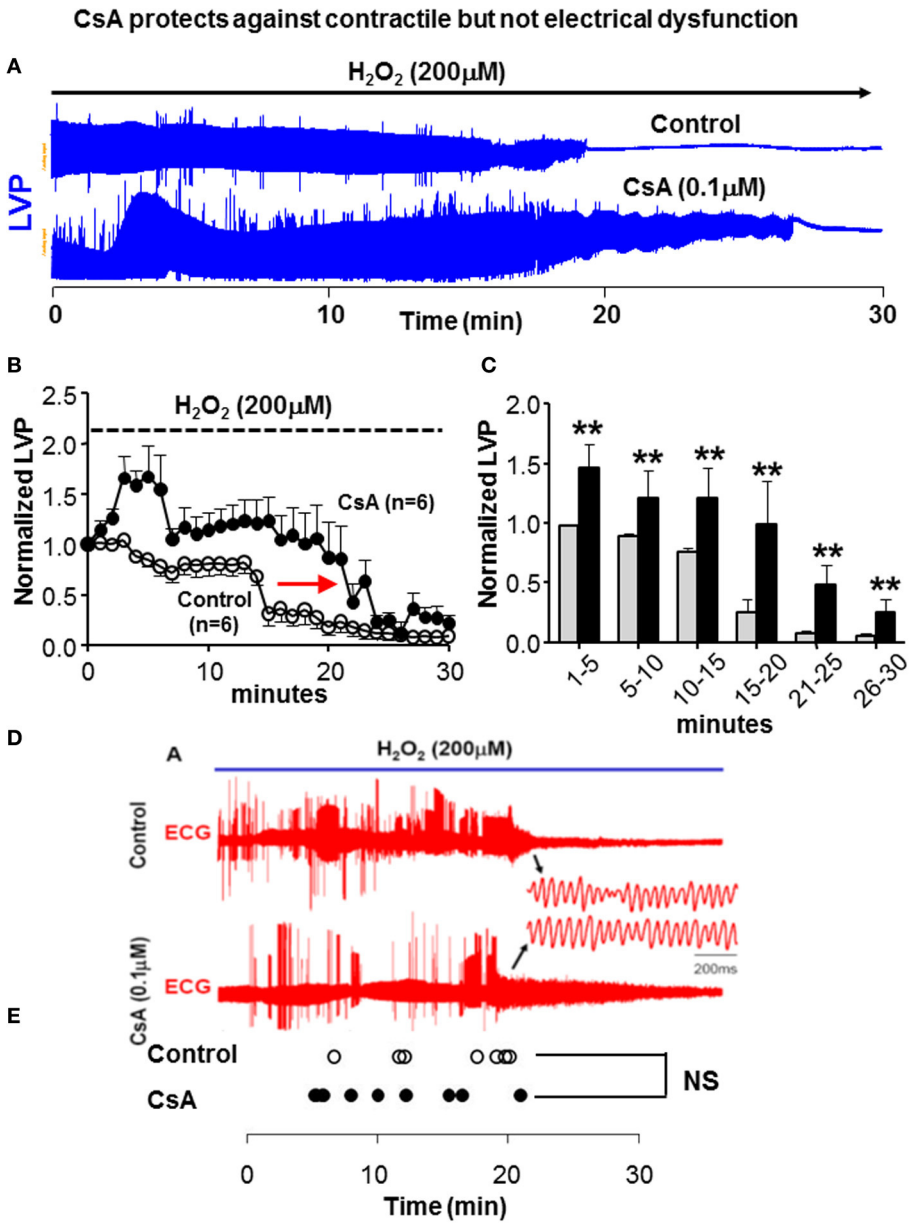

FIGURE 2 | CsA does not protect against electrical dysfunction. (A) Representative time-series of LV pressure waveforms during 30 min of H_2O_2 challenge, indicating reduction and ultimate loss of contractile function. Loss of LVP is delayed in CsA treated hearts. **(B,C)** Average LVP normalized to the baseline pre-H_2O_2 value in all control and CsA hearts. Experiments summarized in **(A–C)** where performed under BDM-free conditions. **(D,E)** ECG traces in control and CsA treated hearts during H_2O_2 challenge and time of onset of VF in all hearts irrespective of BDM presence in the purfase. The transition to VF is not delayed by CsA treatment, indicating lack of electrical protection by CsA. BDM, 2,3-Butanedione monoxime; LVP, Left ventricular pressure; ECG, electrocardiogram; VF, ventricular fibrillation. $^{**}p < 0.01$.

of VF. Surprisingly, we found that CsA treatment failed to protect against the incidence of arrhythmias as the time to onset of VF following H_2O_2 challenge was comparable (p =NS) in control and CsA-treated hearts (**Figures 2D,E**).

PARADOXICAL EFFECT OF CsA

Previously, we and others have highlighted the importance of maintaining $\Delta\Psi_m$ polarization in the protection against OS-induced arrhythmias (Akar et al., 2005; Brown et al., 2010; Lyon et al., 2010). We, therefore, proceeded to investigate the basis for our discrepant findings regarding the role of CsA in protecting against OS-induced $\Delta\Psi_m$ depolarization but not electrical dysfunction. We hypothesized that differences in experimental settings, particularly with regards to the use of the electromechanical uncoupling agent BDM in AP but not $\Delta\Psi_m$ studies may underlie the discrepant outcomes that we observed. Therefore, we repeated our $\Delta\Psi_m$ measurements with and without addition of BDM to the perfusate. Remarkably, we found that BDM

completely reversed the protective effect of CsA on OS-induced $\Delta\Psi_m$ depolarization which we had initially observed (**Figure 3A**, gray background), as CsA-treated hearts exhibited a more rapid $\Delta\Psi_m$ depolarization compared to untreated controls following H_2O_2 challenge when BDM was present in the perfusate (**Figure 3**). As such, the use of BDM revealed a paradoxical effect of CsA which was consistent with exacerbation rather than protection against OS-induced mitochondrial dysfunction. Importantly, BDM alone (i.e., without CsA) did not alter the $\Delta\Psi_m$ response of the heart to OS.

PROTEIN KINASE C INHIBITION ABROGATES THE PROTECTIVE EFFECT OF CsA ON OS-INDUCED MITOCHONDRIAL DYSFUNCTION

BDM is a strong chemical phosphatase, which is known to oppose the phosphorylation of serine/threonine target proteins and to increase ATP depletion in metabolically challenged cardiomyocytes (Stapleton et al., 1998). Therefore, we hypothesized that the paradoxical effect that was unmasked by BDM in terms of CsA-mediated dysfunction may be due to its interference with the activity of the cardioprotective mK_{ATP} channel which is known to modulate mPTP opening at least *in vitro*. Since mK_{ATP} activity is dependent on PKC-mediated phosphorylation, we replaced BDM with the selective PKC inhibitor, CHE. As shown in **Figure 4**, addition of CHE completely abolished the protective effect conferred by CsA. Of note, CHE failed to alter the $\Delta\Psi_m$ response

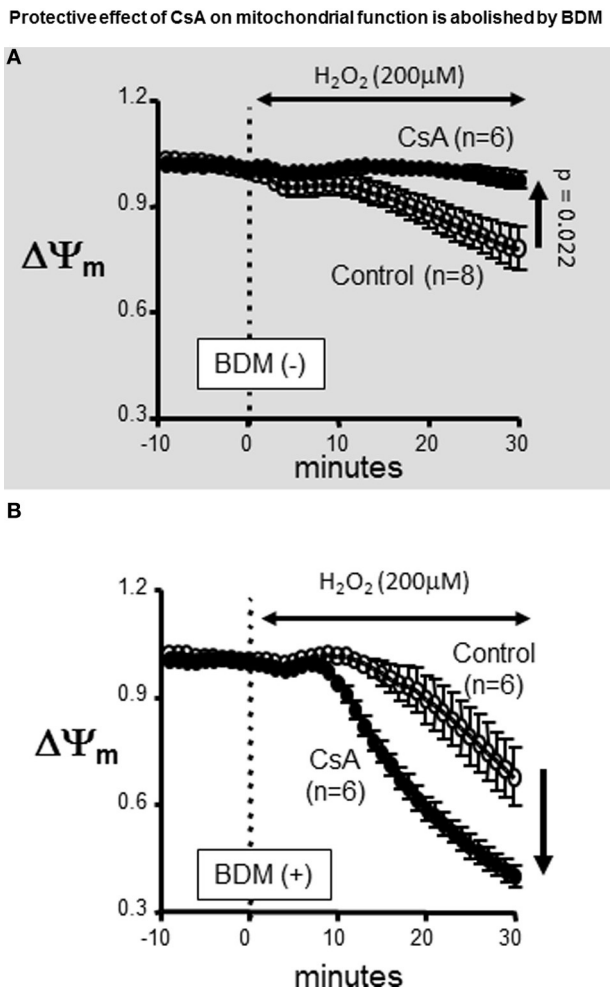

FIGURE 3 | Protective effect of CsA on mitochondrial function is abolished by BDM. Treatment with the chemical phosphatase BDM abolishes the protective effects of CsA. **(A)** For reference, $\Delta\Psi_m$ response presented in **Figure 1** indicating protection by CsA against OS-induced $\Delta\Psi_m$ depolarization. **(B)** Addition of the chemical phosphatase BDM (10 mM) reversed the effect of CsA on $\Delta\Psi_m$. BDM, 2,3-Butanedione monoxime.

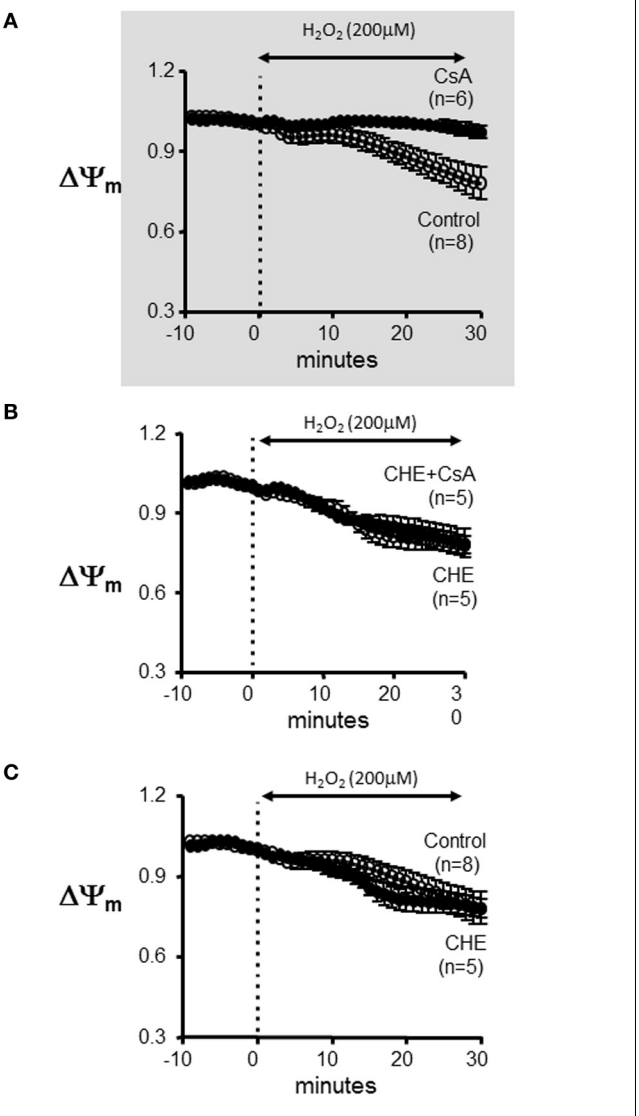

FIGURE 4 | Protective effect of CsA is abolished by PKC inhibition. (A) For reference, $\Delta\Psi_m$ response presented in **Figure 1** indicating protection by CsA against OS-induced $\Delta\Psi_m$ depolarization. **(B)** Average $\Delta\Psi_m$ response to CsA treatment in the presence of the specific PKC inhibitor CHE. **(C)** Average $\Delta\Psi_m$ response in control and CHE treated hearts. CHE alone (i.e., without CsA) did not alter the response. CHE, Chelerythrine; PKC, Protein kinase C.

of the heart to OS when delivered alone (i.e., without CsA) (**Figure 4**, lower panel).

DIRECT mK_{ATP} BLOCKADE ABROGATES THE PROTECTIVE EFFECT OF CsA ON MITOCHONDRIAL DYSFUNCTION

Since PKC has multiple mitochondrial targets that may alter the response of the heart to OS, we tested whether direct pharmacological blockade of mK_{ATP} recapitulated the inhibitory effects of CHE on CSA-mediated cardioprotection. Indeed, addition of 5-HD completely abrogated the protective effect of CsA on $\Delta\Psi_m$ depolarization (**Figure 5**). The $\Delta\Psi_m$ response to H_2O_2 was identical between the control and the combined 5-HD+CsA treated hearts, highlighting the notion that CsA was completely ineffective as a cardioprotective agent under conditions that prevented mK_{ATP} channel activation.

PHARMACOLOGICAL ACTIVATION OF mK_{ATP} PROTECTS AGAINST MITOCHONDRIAL DYSFUNCTION

To further establish the role of mK_{ATP} in the modulation of the $\Delta\Psi_m$ response to OS, we used the pharmacological agonist of the channel, DZX (**Figure 5**). Interestingly, DZX-treated hearts exhibited a markedly blunted $\Delta\Psi_m$ response compared to control hearts; thereby, establishing the efficacy of mK_{ATP} in modulating the opening of mPTP. Once again, the protective effect of DZX on OS-induced $\Delta\Psi_m$ depolarization was prevented by addition of the chemical phosphatase BDM.

PROTECTIVE EFFECT OF CsA ON ARRHYTHMIAS IS DEPENDENT ON mK_{ATP} CHANNEL ACTIVATION

Previously, we and others showed that interventions that stabilized $\Delta\Psi_m$ were associated with protection against post-ischemic arrhythmias, whereas conditions leading to $\Delta\Psi_m$ instability promoted electrical dysfunction (Akar et al., 2005). Therefore, we asked whether modulation of the $\Delta\Psi_m$ response in this model of acute OS could also explain differential vulnerability to arrhythmias. Seven groups were examined in terms of their relative sensitivities to OS-induced mitochondrial dysfunction (quantified by the slope of $\Delta\Psi_m$ depolarization) and electrical dysfunction (assessed by the time to onset of VF). As shown in **Figure 6**, conditions that led to accelerated $\Delta\Psi_m$ depolarization were indeed associated with enhanced vulnerability to VF as they exhibited significantly ($p < 0.05$) shorter time to onset of VF in response to OS challenge. While 11/13 BDM (+) hearts exhibited early (within 15 min) onset of VF, only 1/19 BDM (−)

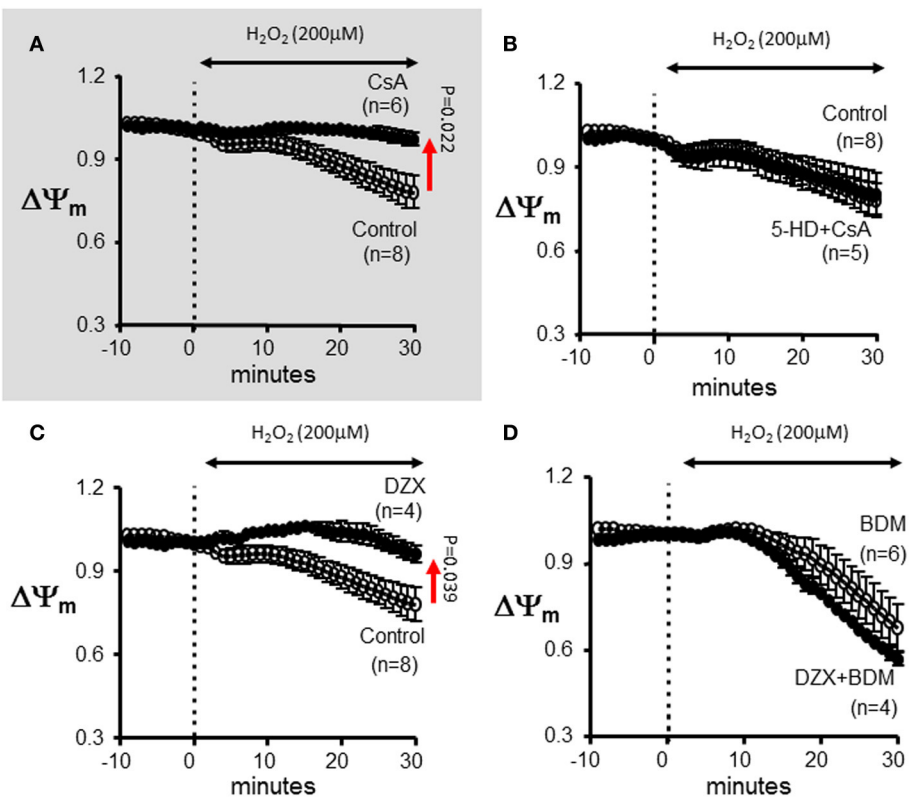

FIGURE 5 | Protective effect of CsA is modulated by mK_{ATP}. (A) For reference, $\Delta\Psi_m$ response presented in **Figure 1** indicating protection by CsA against OS-induced $\Delta\Psi_m$ depolarization. **(B)** Treatment with the mK_{ATP} antagonist abrogates the protective effect of CsA on mitochondrial dysfunction. **(C)** mK_{ATP} activation by DZX protects against OS-induced $\Delta\Psi_m$ depolarization. **(D)** BDM abolishes the protective effect of DZX on mitochondrial dysfunction. DZX, Diazoxide.

hearts were prone to VF within this short time-frame. These findings indicate significantly heightened sensitivity to sustained arrhythmias of hearts treated with the chemical phosphatase ($p = 0.000006$).

To further address the link between mitochondrial and electrophysiological instability and to elucidate the potential mechanism underlying the CsA mediated pro-arrhythmic effect which we uncovered under conditions that prevented mK_{ATP} channel activation and that led to more pronounced $\Delta\Psi_m$ depolarization (**Figure 7A**), we performed detailed optical AP mapping. Analysis of AP properties revealed accelerated shortening of APD in response to OS in CsA-treated hearts compared

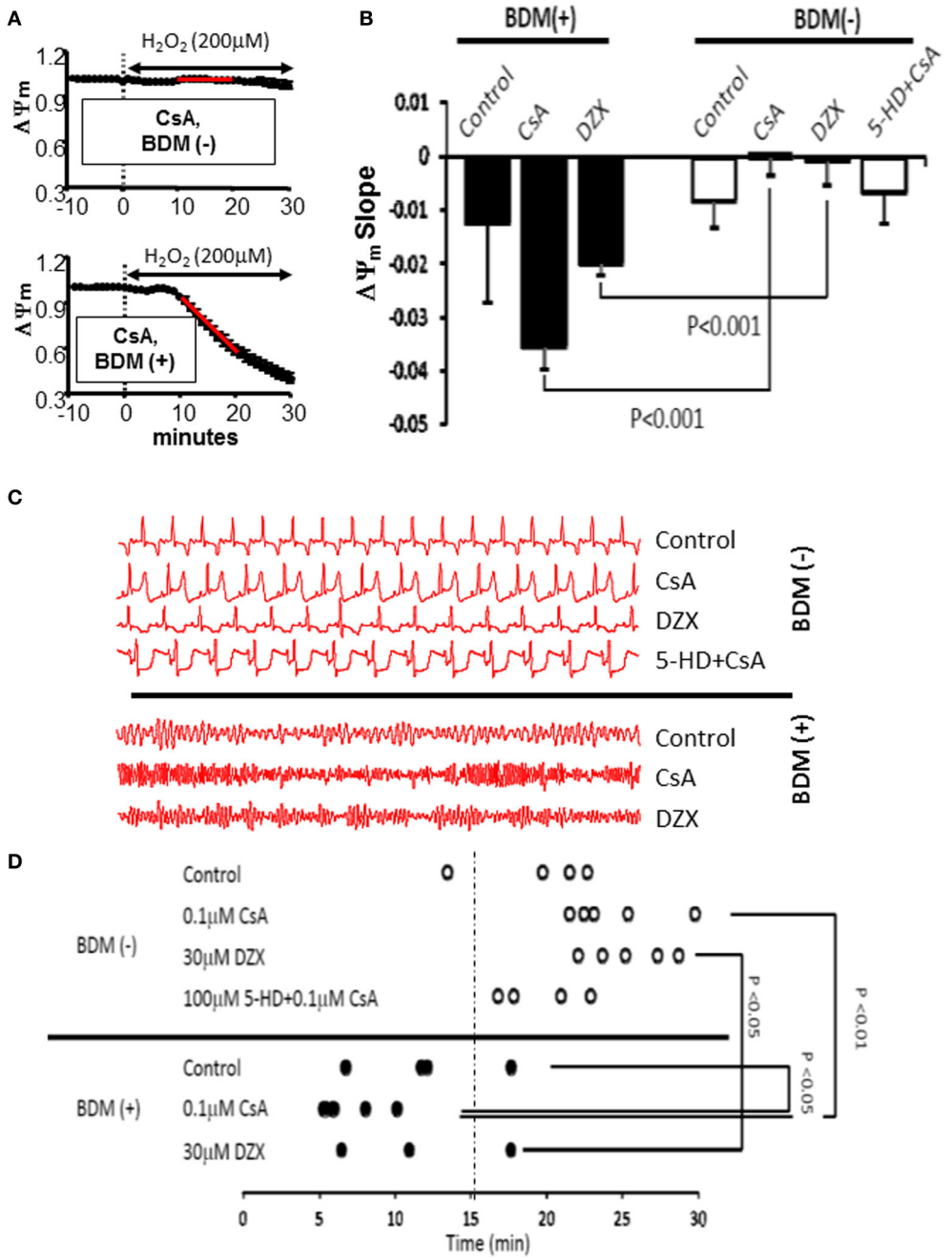

FIGURE 6 | Cross-talk between mK_{ATP} and mPTP modulates mitochondrial and electrical response of hearts to OS. (A) $\Delta\Psi_m$ response to OS in CsA-treated hearts without (top) and with (bottom) concomitant perfusion with the chemical phosphatase BDM. The relative sensitivity of hearts to OS-induced mitochondrial dysfunction was quantified by measuring the slope of $\Delta\Psi_m$ collapse 10–20 min following H_2O_2 perfusion (red line). **(B)** Average slope of OS-induced $\Delta\Psi_m$ change in all groups tested. **(C)** Representative ECG traces from all groups tested, indicating vulnerability to VF in BDM (+) hearts. **(D)** Time to onset of VF following OS challenge as an index of electrical vulnerability in all hearts from all groups. VF, ventricular fibrillation.

to controls (**Figures 7B–E**). These data suggest a heightened electrophysiological sensitivity to OS, particularly with regards to the activation of repolarizing currents as the potential mechanism for CsA-mediated pro-arrhythmia (**Figure 7**).

DISCUSSION

Acute OS manifests in a majority of patients with coronary artery disease, the leading cause of arrhythmic deaths in the United States. Central to the pathology of OS is mitochondrial

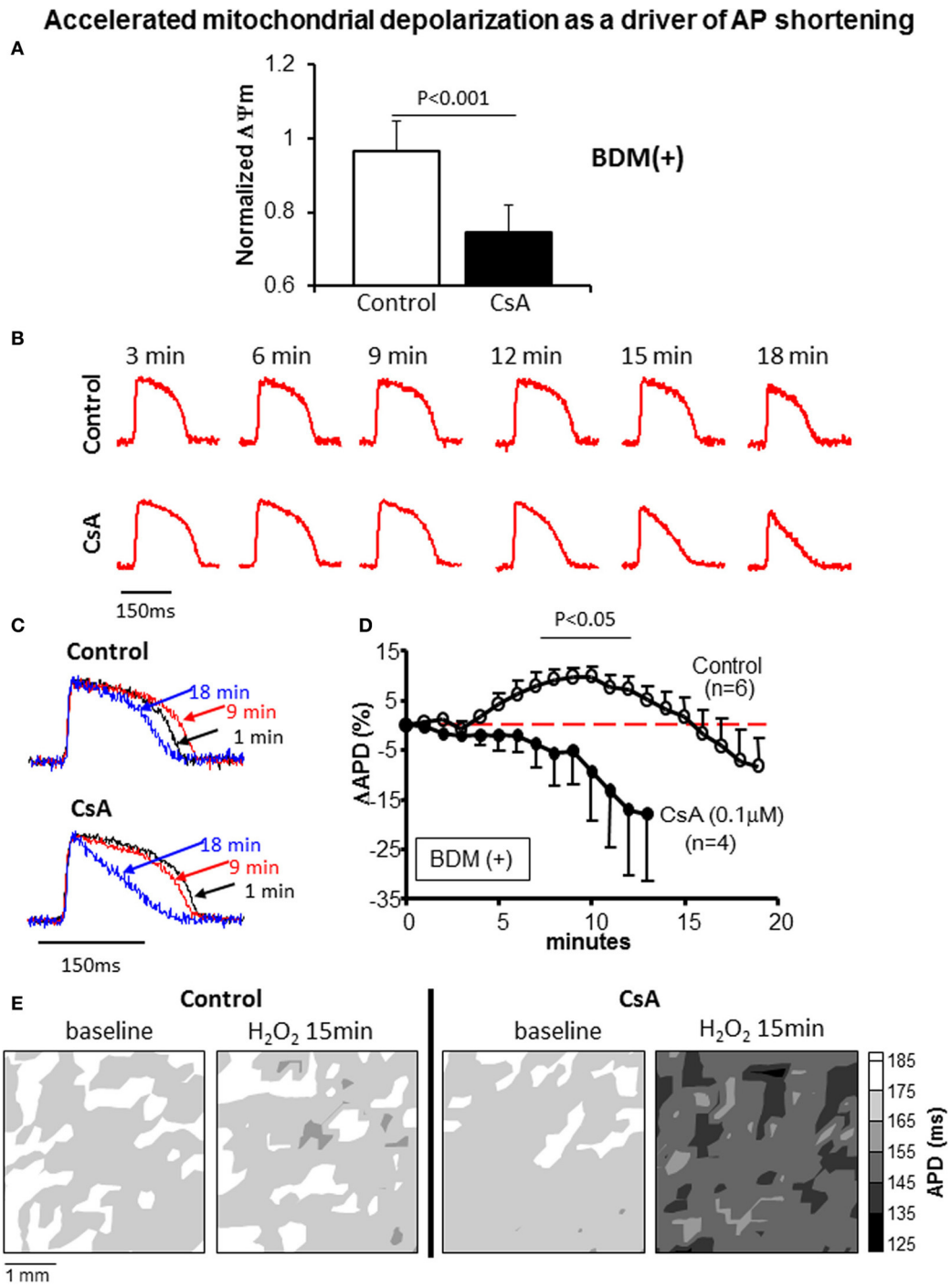

FIGURE 7 | (A) $\Delta\Psi_m$ at 15 min of OS normalized to baseline value in control and CsA treated hearts. **(B,C)** Representative action potentials recorded during early challenge with acute OS in control and CsA treated hearts. AP shortening is more pronounced in CsA compared to control hearts, indicating heightened sensitivity to OS. **(D)** OS-induced % change in APD relative to baseline (pre-H_2O_2 value) in control and CsA treated hearts. **(E)** Representative APD contour maps showing global shortening of APD in response to OS in CsA treated hearts compared to controls.

dysfunction. Although the role of mitochondria as mediators of cell injury is well established, their contribution to arrhythmias is less understood. Indeed, the exact mitochondrial transport pathways that modulate the susceptibility of the heart to electrical dysfunction remain unclear.

In the present work we focused on the mPTP because of its established role in cellular necrosis and MI. Specifically, we investigated the efficacy of CsA in protecting against OS-induced mitochondrial and electrical dysfunction. We chose a simple *ex vivo* model of H_2O_2 challenge which reliably and predictably causes $\Delta\Psi_m$ depolarization and VF within a relatively short (<30 min) time-frame. Our experiments revealed important discrepancies which initially appeared to discredit our central hypothesis that stabilization of $\Delta\Psi_m$ is anti-arrhythmic, as CsA seemed to protect against $\Delta\Psi_m$ depolarization but worsen electrical dysfunction. Further analysis revealed the basis of these discrepant observations, as we uncovered a dual role for CsA in either protecting or impairing cardiac function depending on the cellular milieu. As will be discussed below, our initial findings led us to refine our central hypothesis by examining the functional cross-talk between the mPTP and the cardioprotective mK_{ATP} channels in ultimately mediating the response of the heart to OS. In the present work, we highlight the importance of mK_{ATP} channel availability in determining the efficacy of CsA as a cardioprotective agent.

MITOCHONDRIAL ION CHANNELS AS MEDIATORS OF CARDIAC DYSFUNCTION: ROLE OF mPTP

$\Delta\Psi_m$ is a key metric of mitochondrial function as it forms the proton-motive force used for ATP synthesis. In normal hearts, $\Delta\Psi_m$ is tightly regulated such that ATP synthesis and ROS generation are maintained within a physiological range. In response to OS, $\Delta\Psi_m$ is disrupted, altering over-all energy and redox balance within cardiac myocytes. Specifically, under these conditions, ROS build-up can exceed a threshold level that triggers the sequential opening of mitochondrial channels in a hierarchal manner (IMAC followed by mPTP) (Aon et al., 2007), which in turn leads to $\Delta\Psi_m$ instability. $\Delta\Psi_m$ instability can lead to inexcitability at the cellular level and conduction block and arrhythmias at the organ level, via a mechanism termed "*metabolic sink*" (Akar et al., 2005). Furthermore, pharmacological blockade of IMAC which blunted $\Delta\Psi_m$ depolarization improved electrical and functional recovery of the heart following IR injury (Akar et al., 2005; Brown et al., 2008; Aon et al., 2009). That work, however, focused on relatively mild levels of OS produced by short episodes of IR injury. Since energy dissipating mitochondrial channels exhibit a hierarchal activation pattern in response to rising ROS levels (Aon et al., 2007), we focused in the present work on the efficacy of mPTP blockade by CsA in a model that was tailored to reliably depolarize $\Delta\Psi_m$ and generate VF via MPT formation.

CYCLOSPORIN A AS A CARDIOPROTECTIVE AGENT

The initial success of the immunosuppressive agent CsA in reducing infarct size in patients with coronary artery disease through its potent CycP-D inhibitory activity has fueled considerable interest in its potential use as a therapeutic agent for a wide variety of cardiovascular disorders (Piot et al., 2008; Hausenloy et al., 2012). Despite these encouraging clinical findings, the efficacy of CsA in preventing arrhythmias is unclear (Arteaga et al., 1992; Ko et al., 1997; Schreiner et al., 2004), and recent experimental, preclinical (Lie et al., 2008), and clinical findings (Ghaffari et al., 2013) have cast new doubts regarding the overall utility of CsA. Our current work was designed to directly address issues related to CsA efficacy in improving metabolo-electrical function under conditions of OS.

Consistent with the expected therapeutic benefit of preventing irreversible mPTP opening, CsA treatment in our experiments significantly delayed the onset of OS-induced $\Delta\Psi_m$ collapse and the loss of contractile function in *ex vivo* perfused hearts (**Figures 1**, **2**). However, we found that this metabolo-contractile improvement did not translate into an electrical benefit (**Figure 2D**). Rather, we found evidence of compromised electrical function with no improvement in the onset of VF. As such, our findings regarding lack of arrhythmic protection are fully consistent with those of Artega et al. who reported impairment rather than protection against reperfusion arrhythmias (Arteaga et al., 1992).

Our overarching hypothesis is that stabilization of $\Delta\Psi_m$ is anti-arrhythmic. However, our initial findings regarding improved mitochondrial but not electrophysiological function by CsA appeared to disprove this premise. This prompted us to examine this issue in greater detail. As will be discussed next, our subsequent experiments led to the discovery of an intricate cross-talk within a mitochondrial macromolecular complex that ultimately dictated the functional response of the heart to OS, and conferred upon CsA a dual role as a mediator of protection or dysfunction depending on the specific cellular milieu.

CROSS-TALK BETWEEN mPTP AND mK_{ATP} IN MODULATING $\Delta\Psi_m$ AND ARRHYTHMIAS

A major finding of the present report is the demonstration that the cardiac response to OS is dictated by complex cross-talk between multiple mitochondrial transport mechanisms. Indeed, we found that OS is mediated through an intra-mitochondrial signaling pathway that can either worsen or protect against arrhythmias depending on the nature of its activation. We first showed that BDM, a classical electromechanical uncoupling agent, not only abrogated the protective effect afforded by CsA but rather led to an acceleration of OS-induced mitochondrial depolarization in response to CsA treatment. This paradoxical effect can explain the worsened electrical outcome in terms of APD shortening that we saw upon CsA treatment. Indeed, the synergy between accelerated mitochondrial depolarization and APD shortening is fully consistent with previous cellular and organ level findings (Akar et al., 2005; Aon et al., 2007). It is important to note that BDM alone (without CsA) did not alter the mitochondrial response to OS. This highlights an interaction of BDM with key proteins that modulate the mPTP (the main target of CsA).

Since BDM inhibits phosphokinases and actively dephosphorylates serine/threonine residues on multiple proteins (Stapleton et al., 1998), we hypothesized that the phosphorylation state of a certain target protein which interacts with elements of the

mPTP may be critical for mediating the protective effects of CsA. Given the established role of PKC in mediating cardioprotection by ischemic pre and post-conditioning (Inagaki et al., 2006), we tested whether the detrimental effects of BDM could be explained (at least partially) through its PKC inhibitory activity. We addressed this issue by replacing BDM with the specific PKC inhibitor, CHE. Here too, protection against OS-induced mitochondrial dysfunction by CsA was completely abolished. As such, our findings are consistent with elegant work by the Mochly-Rosen group who highlighted the importance of PKC mediated signaling in the protection against mitochondrial dysfunction in multiple organ systems, including the heart, as well as pioneering molecular and biochemical work from the Garlid laboratory demonstrating the desensitization of mPTP to ROS by PKCε. Unlike BDM, however, CHE did not accelerate (i.e., worsen) the rate of mitochondrial depolarization suggesting involvement of additional off target effects by BDM that adversely impact mitochondrial function. These may include the effects of BDM on a variety of tyrosine kinases as well as its reported role in depleting ATP levels in myocytes under conditions of metabolic challenge (Stapleton et al., 1998). Our present findings not only inform regarding the signaling pathways involved in CsA mediated cardioprotection but also serve to emphasize the need to exercise caution when interpreting findings of studies in which BDM is used, especially those addressing issues relating to metabolic stress.

PKC has numerous target substrates that can impact mitochondrial function either directly or indirectly. One critical target of PKC signaling which has emerged from elegant work by the Marban group and others is the mK_{ATP} channel (Sato et al., 1998). We tested whether direct pharmacological modulation of the channel could potentially explain the detrimental effects of PKC inhibition which we observed. Indeed, we found that 5-HD was as effective as CHE in fully abrogating the protective effects of CsA on OS-induced $\Delta\Psi_m$ depolarization. Although Baines et al. demonstrated that PKCε interacts with multiple key components of the mPTP, including VDAC, ANT, and HKII independently of its interaction with mK_{ATP}(Baines et al., 2003), our present work argues that such mK_{ATP}-independent interactions do not impact the functional response of the intact heart to OS. Indeed, we provide functional data that extend previous *in vitro* studies and give credence to the notion that mK_{ATP} is the central mediator of the cardioprotective effects of PKC on the heart. In light of the importance of mK_{ATP} we went on to investigate the functional consequences of channel activation and found that DZX treatment was as protective as CsA in preventing $\Delta\Psi_m$ collapse. Once again, that protection was prevented by the chemical phosphatase BDM, consistent with the notion that PKC mediated phosphorylation of mK_{ATP} is critical to its opening and therefore efficacy in cardioprotection.

LIMITATIONS

Our study has several important limitations that require mention. For one, we relied upon a pharmacological strategy using DZX and 5-HD to modulate the activity of mK_{ATP}. Although this standard approach which included carefully chosen concentrations was based on numerous published reports, we cannot fully exclude the possibility that minor mK_{ATP}-independent effects may have contributed to our findings.

Moreover, we used a non-ratiometric, semi-quantitative method of TMRM imaging to assess relative (not absolute) changes in mitochondrial function in *ex vivo* perfused hearts. Using this validated method, changes in TMRM fluorescence signal caused by altered cellular membrane potential as opposed to $\Delta\Psi_m$ are negligible.

Finally, we used CsA to inhibit the mPTP. While this is a widely accepted and effective strategy in the heart, Li et al. have shown tissue-specific differences in CyP-D expression and therefore sensitivity to CsA (Li et al., 2012). In particular, they reported that mPTP inhibition in tissues exhibiting low expression of CyP-D, is achieved more effectively using Rotenone than CsA (Li et al., 2012).

CONCLUSION

In summary, our current findings highlight the notion that CsA-mediated cardioprotection against OS requires mK_{ATP} channel activation through a PKC-dependent pathway. Increasing mK_{ATP} activity during CsA administration is required for limiting OS-induced electrical dysfunction. On the other hand, CsA administration during conditions that may prevent mK_{ATP} channel activation may exert unintended pro-arrhythmic consequences through accelerated APD shortening. Our findings may explain existing controversy in the basic and clinical literature surrounding the utility of CsA as a cardioprotective agent.

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Mitochondrial targets for volatile anesthetics against cardiac ischemia-reperfusion injury

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Mitochondria are critical modulators of cell function and are increasingly recognized as proximal sensors and effectors that ultimately determine the balance between cell survival and cell death. Volatile anesthetics (VA) are long known for their cardioprotective effects, as demonstrated by improved mitochondrial and cellular functions, and by reduced necrotic and apoptotic cell death during cardiac ischemia and reperfusion (IR) injury. The molecular mechanisms by which VA impart cardioprotection are still poorly understood. Because of the emerging role of mitochondria as therapeutic targets in diseases, including ischemic heart disease, it is important to know if VA-induced cytoprotective mechanisms are mediated at the mitochondrial level. In recent years, considerable evidence points to direct effects of VA on mitochondrial channel/transporter protein functions and electron transport chain (ETC) complexes as potential targets in mediating cardioprotection. This review furnishes an integrated overview of targets that VA impart on mitochondrial channels/transporters and ETC proteins that could provide a basis for cation regulation and homeostasis, mitochondrial bioenergetics, and reactive oxygen species (ROS) emission in redox signaling for cardiac cell protection during IR injury.

Keywords: volatile anesthetics, isoflurane, mitochondrial bioenergetics, electron transport chain, cardiac IR injury, cardioprotection

INTRODUCTION

In recent years the mitochondrion has gained recognition as a key factor in the etiology of numerous diseases (Duchen, 2004), including cardiac ischemia and reperfusion (IR) injury (Ferrari, 1996; Murphy and Steenbergen, 2008b). Mitochondria act as critical triggers, mediators, and effectors in protective strategies directed against IR injury and other pathological situations (Camara et al., 2010, 2011). Cardioprotective strategies include a complex cascade of signaling events (Zaugg and Schaub, 2003) that not only involve the electron transport chain (ETC) but also key factors in the intrinsic anti-apoptotic signaling pathways that lead to cell protection. Consequently, mitochondria have emerged as regulators of the redox signaling, which is crucial in determining cell fate, i.e., life or death (Brookes et al., 2004).

Cardiac IR-induced mitochondrial dysfunction is accompanied by reduced membrane potential ($\Delta\Psi_m$), decreased adenosine triphosphate (ATP) production, impaired Ca^{2+} homeostasis, increased "bad" reactive oxygen species (ROS) emission, matrix swelling and membrane permeability, and release of cytochrome *c* and other apoptotic factors leading to cell death (Steenbergen et al., 1990; Stowe and Camara, 2009) (**Figure 1**). Pre- and post-conditioning by volatile anesthetics (VA) have emerged as useful strategies to protect the myocardium against IR injury (Zaugg et al., 2003b; Pagel, 2008; Hu and Liu, 2009; Camara et al., 2010). Indeed, the guidelines of the American College of Cardiology and the American Heart Association recommend the maintenance of VA for non-cardiac surgery in patients with increased risk

Abbreviations: VA, volatile anesthetics; IR, ischemia and reperfusion; ETC, electron transport chain; ROS, reactive oxygen species; $\Delta\Psi_m$, mitochondrial membrane potential; MI, myocardial ischemia; CAD, coronary artery disease; OxPhos, oxidative phosphorylation; IPC, ischemic pre-conditioning; PKB, protein kinase B; PKC, protein kinase C; PKA, protein kinase A; ERK, extracellular regulated kinases; APC, anesthetic-preconditioning; RISK, reperfusion injury salvage kinase; CaMK, calcium/calmodulin-dependent protein kinases; GSK, glycogen synthase kinase; RNS, reactive nitrogen species; mK_{ATP}, mitochondrial ATP sensitive K^+-channel; mBK_{Ca}, mitochondrial big Ca^{2+} sensitive K^+ channel; mPTP, kilodalton (kDa), mitochondrial permeability transition pore; mNCE, mitochondrial Na^+-Ca^{2+} exchanger; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; VDAC, voltage-dependent anion channel; PTMs, post-translational modifications; ATP, adenosine triphosphate; IP3, inositol triphosphate receptor; ANT, adenosine nucleotide transport; TCA, tricarboxylic acid; ADP, adenosine triphosphate; NHE, sodium hydrogen exchanger; NCE, sodium calcium exchanger; 5-HD, 5-hydroxydecanoic acid; mCU, mitochondrial calcium uniporter; CyP-D, cyclophilin D; PPIase, peptidylprolyl cis-trans isomerase; CSA cyclosporin A; eNOS, endothelial nitric oxide synthase; APoC, anesthetic-post-conditioning; GPR, G protein coupled receptor; HSP, heat shock protein; ALDH2, aldehyde dehydrogenase 2; 4-HNE, 4-Hydroxynonenal; $Q^\cdot$, ubisemiquinone; UCPs, uncoupling proteins; NF-κB, nuclear factor–κB; O-GlcNAc, O-linked-β-N-acetylglucosamine; miRNA, micro RNA; mDNA, mitochondrial DNA.

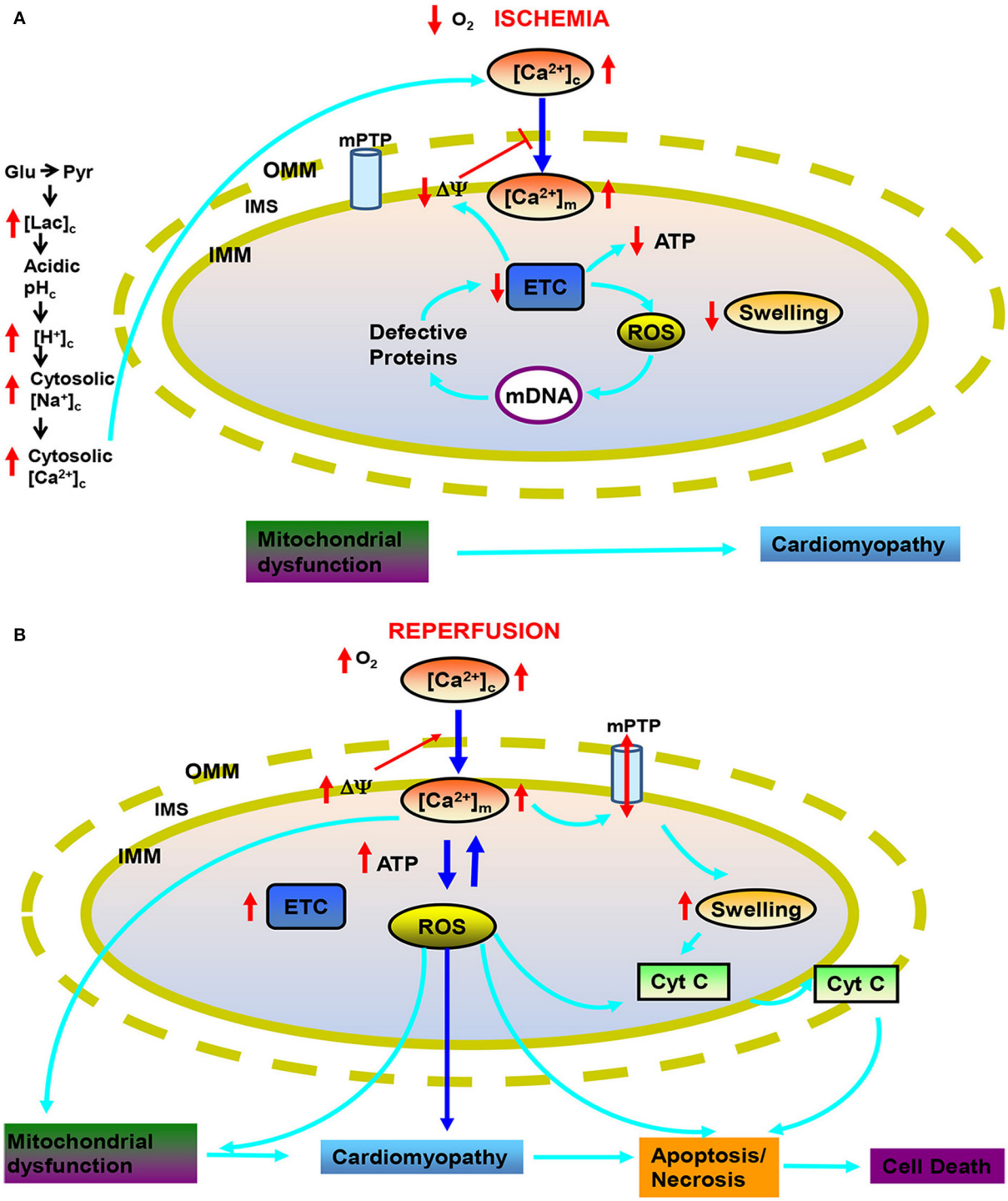

FIGURE 1 | Targets of mitochondria and sequence of changes in cytosolic and mitochondrial function during cardiac ischemia and reperfusion (IR) injury. During ischemia **(A)** reduced O_2 promotes anaerobic glycolysis that generates increased cytosolic lactate (lac_c) leading to acidification. Increased H^+ activates Na^+-H^+ exchanger (NHE) leading to increase cytosolic Na^+ ($[Na^+]_c$), which activates Na^+-Ca^{2+} exchanger (NCE), causing an increase in cytosolic Ca^{2+} ($[Ca^{2+}]_c$) which in turn increases mitochondrial matrix Ca^{2+} ($[Ca^{2+}]_m$). Impaired electron transport leads to increased generation of reactive oxygen species (ROS) beginning with superoxide ($O_2^{\cdot-}$); impaired respiration and substrate utilization leads to uncoupling with lowered mitochondrial membrane potential ($\Delta\Psi_m$) and decreased generation of mitochondrial ATP. During reperfusion **(B)**, the increase in deleterious ROS damages major macromolecules including tricarboxlic acid (TCA) enzymes, membrane transporters, electron transport chain (ETC) proteins and mitochondrial DNA (mtDNA). Also during reperfusion, $\Delta\Psi_m$ is restored and $[Ca^{2+}]_m$ and ROS further increase to produce even greater mitochondria damage that induces mitochondrial permeability transition pore (mPTP) opening and release of cytochrome *c* (cyt C) that in turn triggers apoptosis. Other abbreviations: OMM, outer mitochondrial membrane; IMM, inter mitochondrial membrane; IMS, inter mitochondrial space.

of myocardial ischemia (Fleisher et al., 2007). VA directly target many proteins to modulate their activities, which necessarily complicates analysis of their beneficial effects due to vague structural and dynamic consequences of VA interactions with their target proteins (Eckenhoff and Johansson, 1997). Also, despite advances noted in this review, the complete mitochondrial targets and mechanisms responsible for the protection afforded by VA remain unclear.

This review focuses primarily on the protein targets and functional effects of VA in mediating myocardial protection against IR injury. A special emphasis is given to the direct effects of VA on selected mitochondrial proteins and their implicated mitochondrial mechanisms for myocardial protection against IR injury. There are several cardioprotective strategies or treatments against IR injury directed to mitochondria (Krolikowski et al., 2005; Mewton et al., 2010; Chakrabarti et al., 2013; Jones et al., 2013). Indeed, the cardioprotective effects of VA, which likely include mitochondrial effects, have been tested clinically (Belhomme et al., 1999; Julier et al., 2003; Van der Linden et al., 2003; Zaugg et al., 2003a).

Bridging the gap between bench and bedside should be strengthened by unique therapeutic approaches against IR injury that are targeted to mitochondria. Indeed, because VA as a class are very lipophilic, unlike most other protective drugs, they readily penetrate mitochondria to target the more lipophilic protein sites embedded in the membrane structure. Thus by examining the role of lipophilic agents in mitochondrial-mediated cardioprotection, we may be able to define a new paradigm for mitochondrial protection that could lead to novel approaches to protect the heart in the clinical situation. We hope the information summarized here will provide helpful insights into the potential of synergistic effects of VA at multiple sites in mitochondria that underlie their cardioprotective effects.

MOLECULAR BINDING SITES FOR VA

X-ray crystallography, molecular modeling, and structure–function studies indicate that anesthetics bind in hydrophobic cavities formed within proteins (Bertaccini et al., 2007). The lipophilic (or hydrophobic) nature of these binding sites underlies the Meyer–Overton correlation between anesthetic lipophilicity and potency (Hemmings, 2010). VA exhibit amphiphilicity (possessing both weak polar and nonpolar characteristics), which is required for effective interaction with these hydrophobic cavities, as indicated by a better Meyer–Overton correlation with more polar solvents (Hemmings, 2010). However, identification of anesthetic binding sites on any given target protein is quite difficult due to the low affinity interactions of VA and the paucity of atomic resolution structures for pharmacologically relevant target proteins like membrane bound proteins that are difficult to resolve structurally. In studies using albumin and luciferase, in which 3D atomic resolution structures are available, Bertaccini et al. (2007) found that VA bind in pockets with both nonpolar and polar non-covalent interactions. Binding involves weak hydrogen bond interactions with polar amino acid residues and water molecules, nonpolar van der Waals interactions, and a polarizing effect of the amphiphilic-binding cavity on the relatively hydrophobic anesthetic molecules (Hemmings, 2010).

Internal cavities underlie the conformational flexibility involved in ion channel gating and ligand-induced signal transduction of receptor proteins. Occupation of a critical volume within these cavities by VA provides a plausible mechanism for altering receptor and ion channel function by selective stabilization of a particular confirmation, e.g., an open or inactivated state of an ion channel. VA also acquire binding energy from the entropy generated by displacing bound water from these relatively promiscuous binding sites (Hemmings, 2010). Studies of glycine, $GABA_A$, and NMDA receptors provide convincing evidence for the existence of anesthetic binding sites in critical neuronal signaling proteins by identifying the amino acid residues critical for anesthetic action (Wick et al., 1998; Koltchine et al., 1999; Jenkins et al., 2001; Hemmings, 2010). Although this review centers primarily on VA effects on cardiac mitochondrial protein activities that confer cardiac protection, it is expected that the molecular mechanism for VA action at mitochondrial sites are similar to those for other organelles.

VA AS PHARMACOLOGICAL CONDITIONING AGENTS IN IR INJURY

Clinically, myocardial ischemia (MI) is characterized by a reduced oxygen supply to demand ratio in the hearts of patients at high-risk of coronary artery disease (CAD) or who are undergoing high-risk cardiac surgery. Due to limited blood supply in the manifestation of MI, IR injury leads to a dysfunctional redox imbalance in mitochondria with a concomitant decrease in oxidative phosphorylation (OxPhos) and an overall switch to anaerobic metabolism. Experimental and clinical data have provided several types of mechanical and pharmacological conditioning strategies that lead to reduce IR-induced myocardial dysfunction and cell death as discussed next.

Murry et al. (1986) were the first to coin the concept of ischemic pre-conditioning (IPC), which involves adaptation of the myocardium to longer (damaging) IR stress when preceded by short episodes of repetitive ischemia and reperfusion. IPC evokes many downstream signaling factors (memory) to provide a lasting protection from subsequent lethal index ischemia. Many of the signaling kinases, including Akt/protein kinase B (PKB), protein kinase C-ε (PKC-ε), and extracellular regulated kinases (ERK1/2), translocate to mitochondria to contribute to the acute memory phase in cytoprotection against the impending index ischemia that can lead to heart damage (Zaugg and Schaub, 2003). Administration of a VA before myocardial IR as a protective strategy has been described in different animal and human models (Penta de Peppo et al., 1999; De Hert et al., 2002) as anesthetic-preconditioning (APC) (Tanaka et al., 2004a). APC invokes a memory phase by signaling kinases similar to IPC (Zaugg et al., 2003b). However, the detailed upstream mechanisms of mitochondrial-mediated protection by VA remain unclear.

Clinically, IPC can be mimicked pharmacologically by a variety of substances like aspirin, beta-blockers, alpha 2-adrenoceptor agonists, statins, opioids, and VA (isoflurane, halothane, desflurane, sevoflurane). Myocardial protection by the VA enflurane was first demonstrated by Freedman et al. (1985) in the isolated rat heart, global ischemia model. Later, Warltier et al. (1988) reported that halothane and isoflurane enhanced recovery

of stunned myocardium in dogs during reperfusion. Novalija and Stowe (1998) reported that APC with sevoflurane mimicked IPC by improving vascular, mechanical, and metabolic function in isolated hearts through a sequence of molecular events that ultimately led to protection.

APC has two phases: an acute phase where the initial trigger phase of protection lasts for a few hours, and a delayed phase in which the protection is manifested days after washout of the VA. Although both acute and delayed APC are meditated through complex signal transduction cascades (Weber and Schlack, 2008), acute APC involves phosphorylation and translocation of preexisting proteins, while delayed APC involves *de novo* protein synthesis (Tonkovic-Capin et al., 2002; Tanaka et al., 2004b; Chiari et al., 2005b). APC shares major signaling events with IPC (Zaugg and Schaub, 2003; Zaugg et al., 2003b). That is, like IPC, APC enhances myocardial protection against infarction during early reperfusion by redox activation of protein kinases such as PI3K/Akt (as a part of the reperfusion injury salvage kinase (RISK) pathway), Pim-1 kinase [a member of the family of calcium/calmodulin-dependent protein kinases (CaMK II)], ERK1/2, and by glycogen synthase kinase (GSK-3β) dependent mechanism (Chiari et al., 2005a; Krolikowski et al., 2005, 2006; Weihrauch et al., 2005; Pagel et al., 2006; Stumpner et al., 2012b).

THE MITOCHONDRION AS A TARGET FOR VA PROTECTION IN IR INJURY

Mitochondria serve as the targets and end-effectors for a number of cellular metabolic processes including cell-signaling cascades, redox control, ion homeostasis, cell growth and cell death. In cardiomyocytes they are responsible for generating almost 95% of the cellular ATP; they are also responsible for the majority of the pathological ROS and reactive nitrogen species (RNS) produced during IR. VA likely interfere with mitochondrial function by directly or indirectly targeting many, but not all, mitochondrial proteins. Specifically, VA probably directly modulate the function of known targeted proteins that are believed to underlie the mechanism of IPC-induced protection against IR injury. Therefore, APC is of practical importance because administration of a VA could reduce mortality during ischemic heart surgery, and could also safely be given to patients who are at high risk, e.g., during heart transplant procedure (Ramakrishna et al., 2014). Although the mechanisms for this protection by APC are not well known, it is now evident that the mitochondrion is a key component in the beneficial effects of VA administration (Jovic et al., 2012; Mio et al., 2014).

Based on its pharmacological effects, Kersten et al. (1996) reported that isoflurane-mediated protection against myocardial infarction in dogs involved the putative mitochondrial ATP sensitive K^+-channel (mK_{ATP}), discussed in detail later. VA appear to indirectly relax coronary arteries by altering intracellular Ca^{2+} regulation in the vascular smooth muscle cell by stimulating mK_{ATP} channel opening (Kersten et al., 1996) and/or opening of the mitochondrial big Ca^{2+} sensitive K^+ channel (mBK_{Ca}) (Redel et al., 2008). To date, potential VA-mediated cardioprotective mechanisms targeted to mitochondria involve inhibition of mitochondrial permeability transition pore (mPTP) opening (Pravdic et al., 2010; Sedlic et al., 2010b) (**Figure 2**) via activation of signaling kinases, like PKC (Novalija et al., 2003; Pravdic et al., 2009), mitochondrial uncoupling (Ljubkovic et al., 2007; Sedlic et al., 2009; Pravdic et al., 2012), "small" ROS emission (Tanaka et al., 2002; Novalija et al., 2003; Hirata et al., 2011), inhibition of mitochondrial Na^+-Ca^{2+} exchange (mNCE) (Agarwal et al., 2012), modulation of mitochondrial bioenergetics (Sedlic et al., 2010a; Bienengraeber et al., 2013; Agarwal et al., 2014) (**Figure 2**), and opening of mK_{ATP} (Kersten et al., 1996, 1997; Pain et al., 2000; Stadnicka et al., 2006) and mBK_{Ca} channels (Ozcan et al., 2002; Stumpner et al., 2012a). These diffuse effects of VA on mitochondria may be attributed in part to the complex interactions of the mitochondrial proteins and their association with the membranes that separates the organelle from the cytoplasm and in part to the pleiotropic effects of VA on cell constituents. All of the activators may have a common final pathway, e.g., the triggering of a "small" amount of ROS to stimulate downstream protective pathways.

EFFECTS OF VA ON OUTER MITOCHONDRIAL MEMBRANE PROTEINS

The outer mitochondrial membrane (OMM) contains several enzymes including monomine oxidase and the integral transport proteins, porins, which makes the OMM permeable to small molecules less than 6 kilodaltons (kDa). The voltage-dependent anion channel (VDAC) constitutes the major porin of the OMM and it regulates the metabolic and energetic fluxes across the OMM by transporting metabolites and ions necessary for electron transfer, bioenergetics, and redox potentials for normal mitochondrial function. Mammalian mitochondria have three different VDAC isoforms: 1, 2, and 3 (Craigen and Graham, 2008) that perform different functions (Neumann et al., 2010). Recent reports also suggest a complex regulation of VDAC by mechanisms involving protein-protein interactions and post-translational modifications (PTMs) in normal and pathological conditions (Shimizu et al., 1999; Liu et al., 2009; Das et al., 2012; Porter et al., 2012; Yang et al., 2012). Effects of IR or other oxidative stresses can be exhibited by their ultimate actions on VDAC function. For example, De Stefani et al. (2012) postulated a mechanism by which VDAC permeability promotes apoptosis based on the close anatomic link between VDAC and the inositol triphosphate receptor (IP3) that transfers a large amount of Ca^{2+} from SR to mitochondria during cytosolic Ca^{2+} dysregulation.

The clinical relevance of VDAC in inducing apoptosis (Shoshan-Barmatz and Ben-Hail, 2012) indicates VDAC as a potential target for therapeutic drugs (Shimizu et al., 2001). The increased permeability of VDAC by VDAC oligomerization to create a large pore (Zalk et al., 2005) allows the release of apoptotic factors (e.g., cytochrome *c*), which activate proteolytic enzymes, e.g., the caspases. VDAC normally exists in the open configuration (Hodge and Colombini, 1997), whereas VDAC closure is associated with an increase in oxidative stress and increased Ca^{2+} dependent mPTP transition (Tikunov et al., 2010). The channel gating of VDAC from open to partial closure increased the Ca^{2+} permeability of VDAC (Rostovtseva et al., 2005) so Ca^{2+} imbalance may have a permissive role in mediating the mPTP transition. In contrast, drugs that prevent or impede VDAC closure could have potential therapeutic utility (Vander Heiden

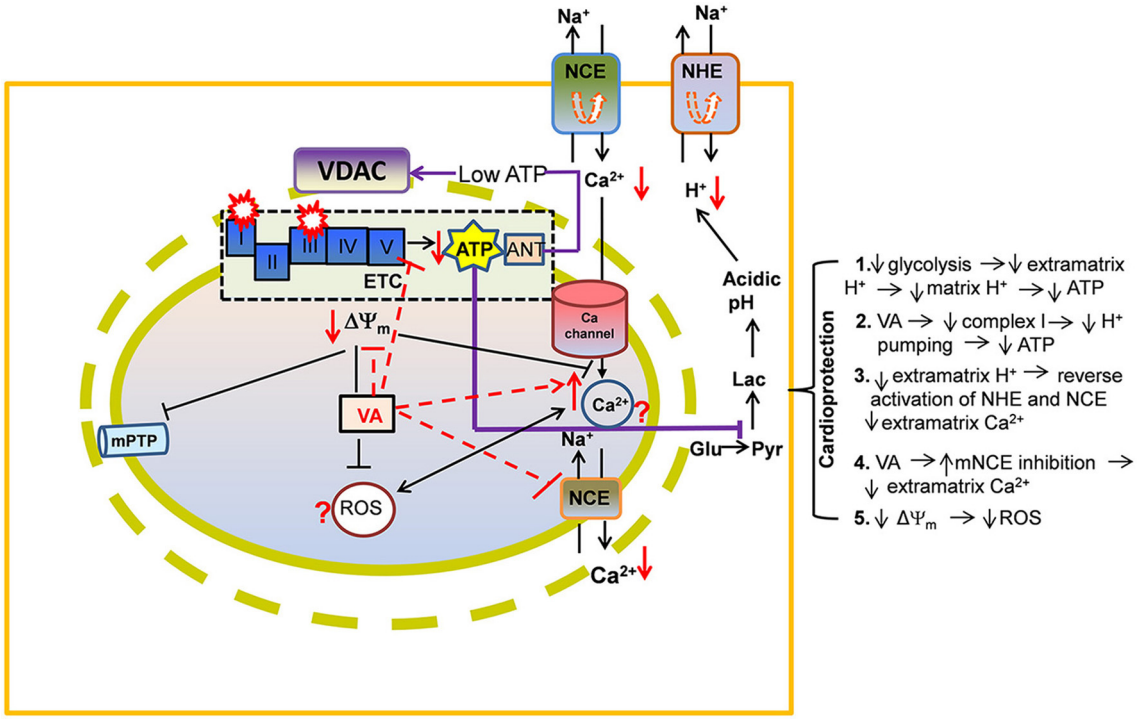

FIGURE 2 | A proposed view of cardioprotection by effects of volatile anesthetics (VA) on electron transport chain (ETC) proteins and on ADP/ATP transport via voltage-dependent anion channel (VDAC). By direct attenuation of NADH dehydrogenase (complex I) and cytochrome bc_1 (complex III), VA promote a slightly more reduced redox state and a slowing of the rates of respiration and phosphorylation. Lowered ATP entry into the matrix through VDAC/ANT may contribute to reduced ATPase activity. These events may help to decrease ATP hydrolysis and so to better maintain cell ATP levels during reperfusion. Preserved ATP synthesis at complex V would diminish the need for glycolysis while decreasing lactic acidosis and cytosolic Ca^{2+} $[Ca^{2+}]_c$ (see details in **Figure 1** legend). Other abbreviations: ROS, reactive oxygen species; mPTP, mitochondrial permeability transition pore; Symbol (↑) represents reverse functioning of NHE and NCE.

et al., 2000; Lemasters and Holmuhamedov, 2006). The modulation of VDAC permeability and release of cytochrome *c* is also regulated by other proteins, such as the Bcl-X protein family, the Bcl-2 homologous antigen/killer (Bak) and Bcl-2 associated X protein (Bax) (Shimizu et al., 1999); however, their definitive roles in pore size modulation is still debatable (Vander Heiden et al., 2000; Shimizu et al., 2001). The downregulation of Bcl-2 and upregulation of Bax protein in myocytes represents the molecular triggers and modulators of apoptotic cell death on reperfusion after ischemia (Zhao et al., 2000).

The anti-apoptotic protein Bcl-2 is also targeted to the mitochondrion and affects different mitochondrial metabolic functions (Imahashi et al., 2004). Isoflurane preconditioning was reported to block the myocardial IR induced decrease in the expression of anti-apoptotic Bcl-2 protein as well as the expression of the pro-apoptotic Bax protein; this led to an increase in the Bcl-2/Bax ratio, mediated through activation of PI3K/Akt signaling (Raphael et al., 2006). VA preconditioning was found to attenuate myocardial cell apoptosis in rabbits after regional IR via Akt signaling and modulation of Bcl-2 family proteins (Raphael et al., 2006). Overexpression of Bcl-2 reduced ischemic injury in hearts by affecting mitochondrial metabolic function as shown by a reduced rate of decline in ATP and enhanced acidification, consistent with Bcl-2 induced inhibition of consumption of glycolytically generated ATP (Imahashi et al., 2004). These effects could have been mediated by reduced entry of ATP into mitochondria via VDAC and/or adenosine nucleotide transport (ANT), or by direct inhibition of F_1F_0 ATPase. (Jamnicki-Abegg et al., 2005) (**Figure 2**) suggested that isoflurane reduces hypoxia-induced apoptosis through activation of Akt and by increased expression of anti-apoptotic Bcl-2 proteins. Thus, accumulating evidence points to a complex regulation of VDAC permeability/gating involving regulation by homo-oligomerization of VDAC, or by hetero-oligomerization with other mitochondrial proteins (e.g., ANT) and extra-mitochondrial proteins (Bak, Bax). Consequently, there are several potential targets for VA to exert their effects in the OMM that may reduce lethal permeabilization of VDAC and provide cardioprotection.

Evidence of VDAC regulation by GSK, the serine/threonine kinase family of proteins involved in glycogen metabolism, provides for an additional role of VDAC in cell injury during IR. Phosphorylation at Ser9 led to inhibition of GSK-3β during preconditioning and this was found to be cardioprotective against IR injury (Nishihara et al., 2006; Gross et al., 2008). The improvement in recovery of perfused rat hearts with a GSK-inhibitor was attributed to decreased ATP translocation through VDAC/ANT, or due to reduced ATP hydrolysis by F_1F_0-ATPase (Das et al., 2008); either pathway is consistent with decreased utilization of

ATP as reported by Murry et al. (1986). The same mechanism of action was proposed to explain the noted improvement in post-ischemic recovery of mice hearts with overexpressed Bcl-2 (Chen et al., 2001). This was supported by another study that showed increased association of VDAC and Bcl-2 during ischemia (Imahashi et al., 2004). Further, the above mechanisms of GSK-dependent fall in ATP translocation into mitochondria was bolstered by a proteomic study that reported alterations in the expressions of ETC proteins during IPC using a GSK inhibitor (Wong et al., 2010) so it is interesting that isoflurane. Isoflurane-induced cardioprotection was also associated with increased levels of phosphorylation of GSK-3β (Zhu et al., 2010).

EFFECTS OF VA ON INNER MITOCHONDRIAL MEMBRANE PROTEINS

The inner mitochondrial membrane (IMM) is impermeable to charged substances and so distinct channels, exchangers, and pumps are utilized to transport ions and metabolites to and from the matrix. The IMM also contains the ETC complexes that carry out OxPhos. This bioenergetic process is dependent on an intricate interplay among the supply of substrates, breakdown and elimination of metabolites, and ion fluxes across the IMM. For example, Ca^{2+} transport into and out of mitochondria is important for buffering excess cytosolic Ca^{2+} and for regulating mitochondrial respiration and ATP production to meet the cellular energetic demands, as in excitation-contraction coupling. Clearly, the mechanisms underlying myocardial contractile dysfunction during and after ischemic insults are due in part to impaired mitochondrial metabolism and ion homeostasis (Bosnjak and Kampine, 1986; Gerstenblith, 2004). A summary of IMM proteins that are affected by VA exposure and their implication in cardioprotection is given in **Figure 3**. VA-induced effects on respiratory complexes are discussed under "Mitochondrial bioenergetics as a target for VA."

VA AND MITOCHONDRIAL Ca^{2+} CHANNELS/TRANSPORTERS IN IR INJURY

Myocardial IR leads to an increase in cytosolic [Ca^{2+}], and consequently to mitochondrial Ca^{2+} loading (Steenbergen et al., 1987), which is a major contributor to mitochondria-mediated necrotic/apoptotic cell injury during IR. APC, like IPC, reduces cytoplasmic Ca^{2+} load and improves myocardial Ca^{2+} responsiveness so that reperfusion injury is attenuated (An et al., 2001). A detailed mechanistic understanding of this process remains to be explored. However, Riess et al. (2002b) reported that APC-mediated cardiac protection against Ca^{2+} overload on reperfusion was blocked by a putative mK_{ATP} inhibitor (5-hydroxydecanoic acid; 5-HD), suggesting that mK_{ATP} channel opening was associated with a decrease in matrix Ca^{2+} overload possibly via attenuated Ca^{2+} uptake.

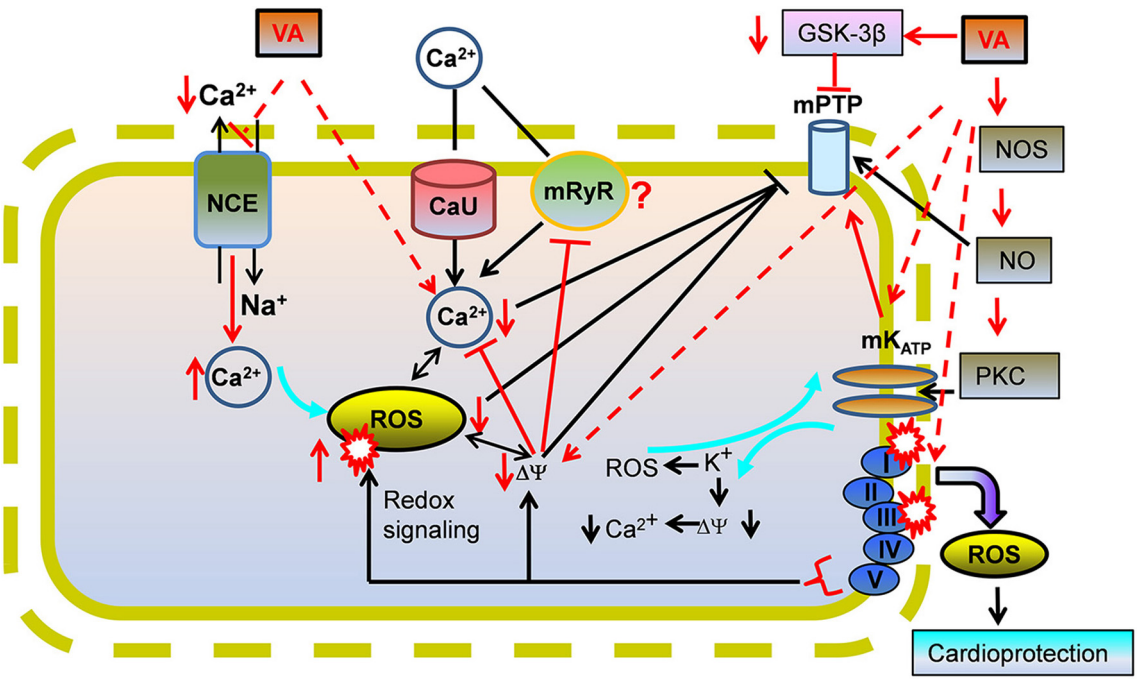

FIGURE 3 | A proposed view of cardioprotection by effects of volatile anesthetics (VA) on mitochondrial Ca^{2+} overload. VA could mediate cardioprotection by mildly inhibiting mitochondrial NCE to increase $[Ca^{2+}]_m$ which triggers protective mechanisms before IR injury. Lowered ATP or higher Ca^{2+} -induced stimulation of mitochondrial K^+ channels may lead to mild uncoupling by the K^+-H^+ exchanger (KHE) that may reduce $\Delta\Psi_m$ and $[Ca^{2+}]_m$ during IR via the mitochondrial Ca^{2+} uniporter (CU) and/or the putative mitochondrial ryanodine receptor (mRyR). Lowered $[Ca^{2+}]_m$ would decrease "deleterious" ROS emission, impede mPTP opening, and reduce apoptotic/necrotic cell death on reperfusion. mPTP opening could also be prevented by a VA-mediated decrease in activation of glycogen synthase kinase (GSK-3β) via phosphorylation of GSK-3β. Effects of VA on channels/exchangers suggest potential implications for low Ca^{2+} and ROS in the triggering phase of VA cardioprotection.

The uptake of Ca^{2+} by the mitochondrial calcium uniporter (mCU) specifically depends on a large $\Delta\Psi_m$ gradient (Saotome et al., 2005). Therefore, slight $\Delta\Psi_m$ depolarization represents a strategy for cardioprotection. In a recent study we (Agarwal et al., 2012) found that direct exposure of mitochondria to isoflurane at a physiological Ca^{2+} concentration (∼200 nM free) led to a Na^+-dependent, but $\Delta\Psi_m$-independent, increase in mitochondrial Ca^{2+} by attenuating NCE without affecting uptake via the mCU. Moreover, this was consistent with the lack of increase in matrix Ca^{2+} in the absence of buffer Na^+ so that NCE could not be activated (Agarwal et al., 2012). These observations are compatible with a study by Sedlic et al. (2010b), in which a small increase in Ca^{2+} uptake was found in mitochondria isolated from isoflurane preconditioned rat hearts, even though mild loss of $\Delta\Psi_m$ occurred. The decrease in $\Delta\Psi_m$ also attenuated deleterious ROS production and attenuated mPTP opening. However, despite a fall in $\Delta\Psi_m$ and a decrease in ROS emission, isolated mitochondria can still exhibit a small rise in matrix Ca^{2+}, so there are exceptions to the idea that a rise in matrix Ca^{2+} is only a consequence of ROS-induced Ca^{2+} release phenomenon (Zima and Blatter, 2006). In our study (Agarwal et al., 2012), we speculated that the small increase in matrix Ca^{2+} induced by isoflurane could be part of the trigger mechanisms that include ROS in the signaling cascades that underlie VA cardioprotection. This notion remains to be verified experimentally. If proven, it could provide a novel insight into the triggering role of matrix Ca^{2+} in APC.

VA AND MITOCHONDRIAL K_{ATP} (mK_{ATP}) CHANNELS IN IR INJURY

mK_{ATP} channels are thought to be located in the IMM, and like other mK^+ channels with different ligands, are widely recognized as redox sensors of ischemia (e.g., low ATP, high Ca^{2+}, low pH) that trigger effectors of several survival signaling pathways involved in pre- and post-conditioning (Gross and Fryer, 1999). In spite of numerous electrophysiological and pharmacological approaches to discern the molecular identity and composition of the mK_{ATP} channel, the true identity remains contentious. Using elaborate experimental approaches involving unbiased proteomic and pharmacological techniques, the KCNJ1 (ROMK) was identified in the IMM and demonstrated that ROMK channels can localize to mitochondria (Foster et al., 2012). The channel was shown to mediate ATP-sensitive K^+ flux and to confer cytoprotection. However, as noted by Wojtovich et al. (2010) the assignment of ROMK as the mK_{ATP} conflicts with pharmacological data on the sensitivity of either channel to ATP and fluoxetine.

Nonetheless, mK_{ATP} channel opening, or any other mK^+ channel, may be an important component of mitochondrial and cellular protection against cardiac IR injury. The cause-effect relationships of the components that lead to protection, however, are unclear. APC was reported to cause production of a small amount of ROS/RNS (Kevin et al., 2005), which could activate certain intracellular signals, like $NO^{\cdot}$, that led to activation of the mK_{ATP} channel (Novalija et al., 1999). Putative mK_{ATP} channel openers led to mild swelling and uncoupling of mitochondria (mild loss of $\Delta\Psi_m$), and a "small" transient rise in ROS emission (signaling ROS) associated with a decrease in mitochondrial Ca^{2+} load (Wang et al., 2001; Facundo et al., 2006a,b). VA exert cardioprotective effects that most certainly involve mitochondrial bioenergetics (discussed later), and also mK_{ATP} channel (or other K^+ channels) opening, as reviewed by our group previously (Riess et al., 2004b; Stowe and Kevin, 2004; De Hert et al., 2005; Kevin et al., 2005).

Several other reports support the association of VA and K_{ATP} channels on mitochondrial function. Jiang et al. (2007) reconstituted fragments of the IMM from human left ventricle, and based on use of the putative mK_{ATP} channel antagonist, 5-HD, they reported that isoflurane increased the open probability of the putative mK_{ATP} channel. Similarly, H_2O_2 was able to activate the putative mK_{ATP} channel; this finding was supported by a similar study (Queliconi et al., 2011). These data confirm that isoflurane, as well as ROS, directly modulate reconstituted cardiac mK_{ATP} channels without apparent involvement of cytosolic protein kinases, as commonly proposed. Sevoflurane preconditioning protected the myocardium against IR injury by reducing mitochondrial Ca^{2+} loading, again presumably via mK_{ATP} channel opening (Wang et al., 2001; Chen et al., 2002; Liu et al., 2005). Sevoflurane induced cardioprotection was also proposed to increase mitochondrial volume via mK_{ATP} channel opening on the basis of effects of putative agonists and antagonists on K_{ATP} channels (Riess et al., 2008b). Desflurane prevented mPTP opening and this effect was abrogated by pretreatment with a mK_{ATP} channel antagonist, which suggested a link between mPTP opening and mK_{ATP} channel activation during cardioprotection (Riess et al., 2002b, 2003, 2008a; Piriou et al., 2004). However, the mechanism regulating mK_{ATP} dependent mPTP transitions still remains to be verified. Moreover, the sensitivity and selectivity of 5-HD and diazoxide as modulators of mK_{ATP} channels have been questioned (Hanley et al., 2002; Lim et al., 2002; O'Rourke, 2004). Thus, although these studies suggest overall that VA act on mK_{ATP} channels as a mechanism to contribute to cell protection, the effects could have been on other mK^+ channels or due to other upstream mechanisms.

VA AND MITOCHONDRIAL PERMEABILITY TRANSITION PORE (mPTP) IN IR INJURY

The mPTP is a non-specific channel that allows water, ions, and solutes with low molecular weights (≤1.5 kDa) to traverse mitochondrial membranes and enhance ROS emission, mitochondrial swelling and cell death. The molecular identity of the mPTP remains unclear. The mitochondrial matrix protein, cyclophilin D (CyP-D), a member of a family of highly homologous peptidylprolyl cis-trans isomerase (PPIase), is believed to constitute an integral component of the mPTP, and thus to play an important role in regulating the pore (Nicolli et al., 1996). Previously it was suggested that the VDAC-ANT-CyP-D complex constituted the structural and functional component of the mPTP by its sensitivity to cyclosporin A (CsA), an inhibitor of the pore (Crompton et al., 1998). However, subsequent genetic loss- and gain-of-functional studies have questioned the relevance of VDAC in the formation of mPTP (Javadov et al., 2009; Bernardi, 2013). Interestingly, in a recent study, the F_1F_0-ATP synthase was proposed to be the mPTP, or at least a component of the mPTP complex (Giorgio et al., 2013). Increased

mitochondrial ROS, in addition to Ca^{2+} overload, alkalosis, and ATP depletion (Halestrap, 2010), are major hallmarks of IR injury and are some of the primary factors that lead to mPTP opening.

IR-activated pathways of cell death are likely mediated by mPTP because CsA and sanglifehrin A, inhibitors of the pore, were found to reduce infarct size (Clarke et al., 2002). Thus, preventing mPTP opening serves as a clinically relevant therapeutic target for treating IR injury. VA-induced cardioprotection is associated with reduced mPTP opening (Krolikowski et al., 2005; Pravdic et al., 2009; Sedlic et al., 2010b). $NO^{\cdot}$ produced by endothelial $NO^{\cdot}$ synthase (eNOS) during cardioprotection by anesthetic post-conditioning (APoC) was suggested to prevent mPTP opening (Ge et al., 2010). Sevoflurane, like CsA, increased the threshold of Ca^{2+}-induced mPTP opening when mediated via GSK-3β inactivation (Onishi et al., 2012). Moreover, the interaction of PKC-ε and the putative constituents of the pore (VDAC, ANT) suggested that a signaling mechanism could modulate mPTP function (Baines et al., 2003). Isoflurane preconditioning reduced cytochrome *c* release (Qian et al., 2005), possibly by activating a PKC-3-dependent mechanism linked to retarded mPTP opening. Isoflurane was found to induce phosphorylation of GSK-3β, which was associated with mitochondrial protection and reduced IR injury due to attenuated mPTP opening (Juhaszova et al., 2004). Moreover, phosphorylation of GSK-3β was reported to increase binding of ANT with phosphorylated GSK-3β (Nishihara et al., 2007), which decreased binding of ANT with CyP-D and suppressed mPTP formation to ultimately confer cardioprotection (Hausenloy et al., 2002; Javadov et al., 2003). Future confirmation of the molecular identity of mPTP is indispensable to understanding VA-mediated mechanisms that would potentially retard mPTP opening and confer cardioprotection. An isoflurane-mediated decrease in ROS production inhibited earlier opening of the mPTP and reduced apoptosis (Wu et al., 2014) during hypoxia/reoxgenation in isolated cardiomyocytes.

MITOCHONDRIAL BIOENERGETICS AS A TARGET FOR VA

FUNCTION OF MITOCHONDRIAL ELECTRON TRANSPORT CHAIN (ETC) COMPLEXES

Mitochondria regulate metabolism in addition to synthesizing ATP. Mitochondrial dysfunction underlies various pathological processes, including IR injury, as emphasized in this review. Consequently, preservation of mitochondrial function is necessary to abrogate mitochondrial energy imbalances and apoptotic signaling pathways that occur in IR injury (Chen et al., 2007). Delineating the underlying molecular mechanisms that act either as triggers, activators, or end-effectors is crucial for understanding the complex cardiac protective vs. detrimental mechanisms mediated by mitochondria. An understanding of how VA alter mitochondrial bioenergetics is highly significant because mitochondrial respiratory dysfunction is reportedly a trigger of IR injury and VA are cardioprotective. The scheme representing known VA targets of ETC proteins and their modulating effects on ETC functions are summarized in **Figure 4**.

VA are well known to mediate myocardial protection in part by attenuating the activity of ETC proteins, the first of which is complex I (Riess et al., 2002a, 2005). Attenuating activity of complex I appears to produce a small transient increase in ROS, which could then serve as a trigger for cellular protection (Kevin et al., 2003; Riess et al., 2004a). During oxidation of complex I substrate, complex III is considered the principal source for ROS generation in isolated mitochondria; but this can be inhibited by limiting electron flow from complex I to complex III (Chen et al., 2003; Aldakkak et al., 2008). A sevoflurane-mediated decrease in complex I activity was reversed with ROS scavengers; this suggested that the trigger in protection involves modulation of ETC complexes and generation of ROS (Riess et al., 2004a).

In our most recent study (Agarwal et al., 2014) we explored the potential ETC protein targets of isoflurane by comparing its effects with known ETC inhibitors. We found a differential modulation of NADH, $\Delta\Psi_m$, and respiration by isoflurane under different substrates conditions. We furnished inferential evidence that isoflurane directly attenuates forward and reverse electron flow, in a substrate dependent manner, by selectively inhibiting ETC complexes I and III. Complexes II, IV, and V, as well as ANT activities were unaffected by isoflurane. These results supported some selectivity of isoflurane in its interaction with different mitochondrial proteins. With the complex I substrate, pyruvate/malate, isoflurane decreased the magnitude of state 3 NADH oxidation, increased transient state 3 depolarization, and depressed state 3 respiration by attenuating complex I in a manner similar to low concentrations (nM) of a complex I inhibitor (rotenone).

Limiting complex I activity during ischemia has the potential to minimize ROS accumulation on reperfusion and so to protect mitochondria and cells from oxidative damage (Aldakkak et al., 2008). With the complex II substrate succinate, isoflurane only slightly reduced NADH oxidation, $\Delta\Psi_m$ depolarization and state 3 respiration (Agarwal et al., 2014). In the presence of succinate and inhibition of complex I with rotenone, isoflurane increased the rates of state 3 and 4 respiration by attenuating complex III activity. Attenuated electron transfer at complex III leads to electron leak and ROS generation. Thus, the cardioprotective effect of VA against IR injury could be triggered by a small rise in ROS, which can occur with modulation of the activity of ETC complexes. This is supported by a study (Ludwig et al., 2004a), in which an isoflurane-induced small increase in ROS and reduction in myocardial infarct size *in vivo* were attenuated by a complex III inhibitor, but not by a complex I inhibitor. This study suggested that ROS generation from complex III at some point during IR injury is a crucial intracellular redox mediator of isoflurane-induced preconditioning.

The generation of a "small" transient signaling ROS from the ETC most likely originates from the ubisemiquinone ($Q^{\cdot}$) radical intermediate via electron transport in complex III (Chen et al., 2003) and so this may be a crucial mediator of VA-induced conditioning (Kevin et al., 2003; Ludwig et al., 2004a). Hirata et al. (2011) reported that isoflurane increased the generation of signaling ROS at complexes I and III, and decreased the reverse electron flow -mediated detrimental ROS generation, by attenuating complex I activity during reperfusion. Thus, attenuation of mitochondrial complexes I and III by VA may trigger the signaling ROS that, via downstream pathways, decrease production of

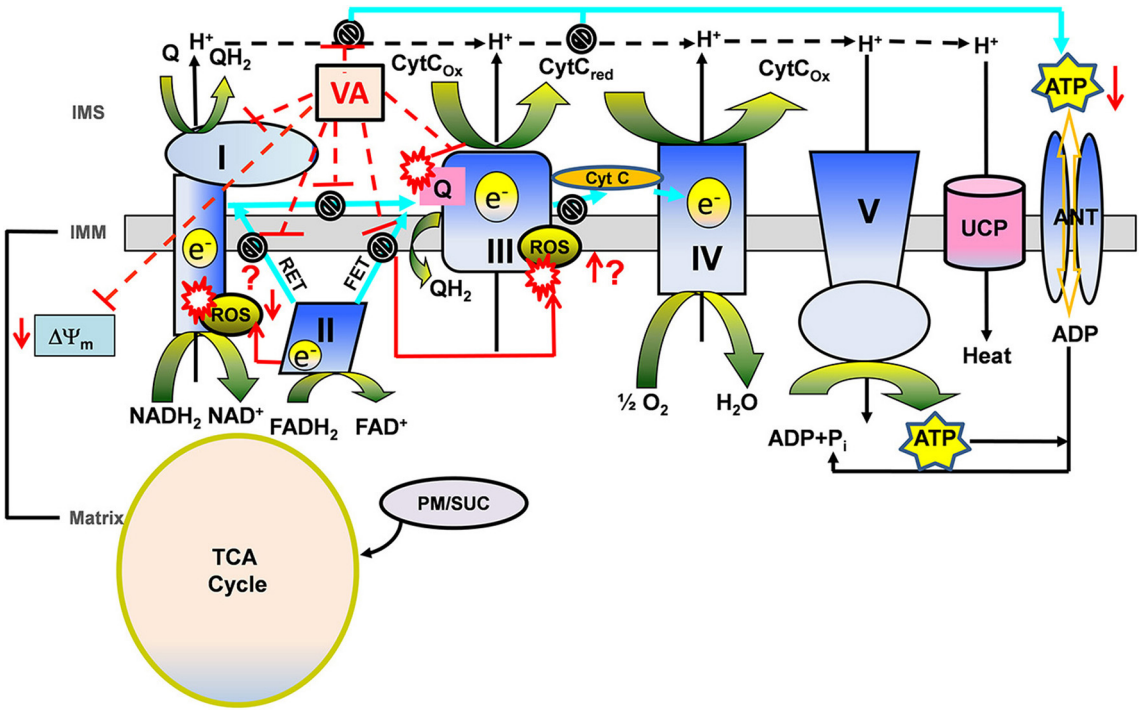

FIGURE 4 | A proposed view of cardioprotection based on the modulating effects of volatile anesthetics (VA) on electron transport chain (ETC) function in the presence of different substrates. Mitochondria generate reducing equivalents, $NADH_2$ and $FADH_2$, via the tricarboxylic acid (TCA) cycle in the matrix during electron transfer through the ETC complexes of the inner mitochondrial membrane (IMM) to generate the proton gradient (H^+) and mitochondrial membrane potential ($\Delta\Psi_m$). The transfer of electrons through the ETC complexes to the final electron acceptor, O_2, is coupled with the H^+ gradient to phosphorylate ADP to ATP by ATP synthase (complex V). During electron transfer, attenuation of complex I and complex III generates O_2 free radical anions ($O_2^{\cdot -}$) leading to other ROS. Complex II mediates forward and reverse electron transfer (FET and RET, respectively), which generates ROS at complex I and complex III. VA modulate complex I and III, therefore affecting bioenergetics ($\Delta\Psi_m$, redox state, respiration, phosphorylation). During oxidation of complex II substrate succinate (Suc), ROS generated via RET may be considered "deleterious," while ROS generated via FET by complex III inhibition may represent the "signaling" ROS. VA-induced complex I inhibition may decrease the generation of RET mediated "deleterious" ROS and could mediate the generation of "signaling" ROS at complex III. Whereas, during oxidation of complex I substrate pyruvate/malate (PM), VA-induced complex I inhibition could mediate generation of "signaling" ROS at complex I. VA-induced complex I and complex III inhibition may mediate slower rates of proton and electron transfer to reduce ATP synthesis during ischemia to preserve it during reperfusion. Possible modulating effects of VA on uncoupling protein (UCP) in promoting proton leak and uncoupling in cardioprotection are also noted. Other abbreviations: ANT, adenine nucleotide translocase; TCA cycle, tricarboxylic acid cycle; IMS, inter mitochondrial space.

deleterious ROS from these complexes and confer cardioprotection. In the isolated heart model, APC also may be triggered by the formation of a small transient amount of RNS, because decreased cardioprotection was found during VA exposure with application of either a ROS scavenger or a $NO^{\cdot}$ inhibitor (Novalija et al., 2002). VA also enhanced myocardial recovery during reperfusion by opposing the adverse effects of deleterious ROS on cardiac function (Tanguay et al., 1991). It is also possible that aside from the direct modulatory effects of VA on ETC complexes that leads to slight increase in the "triggering" ROS production, VA could mediate their effects on ROS generation through modulation of the ROS balance, i.e., generation vs. scavenging, by affecting the mitochondrial antioxidant defense mechanism (Nickel et al., 2014).

VA AND MITOCHONDRIAL MEMBRANE POTENTIAL

The active pumping of protons (H^+) from the matrix to the intermembrane space generates the $\Delta\Psi_m$ necessary for OxPhos. A H^+ leak (uncoupling) in the IMM has potential implications in both IR injury and in preconditioning (Nadtochiy et al., 2006). Murphy et al. (2003) suggested that exposure to ROS mediates activation of uncoupling proteins (UCPs), which tend to reduce $\Delta\Psi_m$ and hasten respiration. A lowered mitochondrial pH and $\Delta\Psi_m$ may be markers of VA-induced cardioprotection (Pravdic et al., 2010). The direct effect of isoflurane to decrease $\Delta\Psi_m$ due to reduced complex I activity and increased mitochondrial acidification via an ATP synthase-mediated increase in proton influx (Pravdic et al., 2012) is another alternative mechanism for VA-induced cardioprotection. Preconditioning may increase ROS production due to flavoprotein oxidation and mitochondrial uncoupling while the decrease in $\Delta\Psi_m$ may be coupled to mK_{ATP} channel opening (Ljubkovic et al., 2007; Sedlic et al., 2009).

Sedlic et al. (2010a) examined isoflurane's site of action in the ETC using an isolated rat cardiomyocyte model and reported an uncoupling-induced depression of $\Delta\Psi_m$ and complex I inhibition by isoflurane as two potential mechanisms contributing to protection against IR injury. It was reported that UCP-3 protected the heart against IR injury and that UPC-3 knockout mice lost the

cardioprotection conferred by IPC (Ozcan et al., 2013). However, Pravdic et al. (2012) reported that isoflurane, like UCPs, caused a mild depolarization and matrix acidification possibly by reducing complex I function and increasing H^+ flux through ATP synthase; they also reported that UCPs appeared not to be involved in APC. A decrease in $\Delta\Psi_m$ and reduced mitochondrial Ca^{2+} uptake, with concomitant tolerance to hypoxia-reoxygenation, was reported to occur in isolated cardiomyocytes and mitochondria examined after cardiac preconditioning in rats (Ljubkovic et al., 2007). In another study, an APC-induced decrease in $\Delta\Psi_m$ was reported to be beneficial in decreasing excess ROS emission and mitochondrial Ca^{2+} accumulation in oxidatively stressed cardiomyocytes; this effect was suggested to be due to a mild uncoupling effect (Sedlic et al., 2010b). However, in many of these studies the physiological relevance of mild uncoupling by VA, if it occurred, is questionable (Shabalina and Nedergaard, 2011) because the depression of $\Delta\Psi_m$ was observed only in isolated mitochondria metabolizing a high concentration of succinate (Shabalina and Nedergaard, 2011) and exhibiting a large increase in respiration (Agarwal et al., 2014). Moreover, it is possible that the mild uncoupling was mediated by an effect of VA to activate other mK^+ channels. In any case, the molecular basis of VA interactions with the mitochondrial ETC proteins that leads to decreased or unchanged $\Delta\Psi_m$ is remains unclear.

Oxidative phosphorylation is central to substrate metabolism and energy production. Wide variations in OxPhos rates occur to match workload demand to ATP supply. This rate is regulated in part by Ca^{2+}-induced activation of several TCA enzymes; but other factors are also involved (Vinnakota et al., 2011; Boelens et al., 2013). On reperfusion, APoC was shown to depress mitochondrial respiration, to partially depolarize mitochondria, and to decrease mitochondrial pH (Pravdic et al., 2010). These events led to retarded mPTP opening and, consequently to better preserved $\Delta\Psi_m$ and ATP synthesis, and reduced intracellular and mitochondrial Ca^{2+} overload and cell death (Pravdic et al., 2010). Isoflurane pre- and post-conditioning was reported to induce phosphorylation of mitochondrial proteins, with ANT phosphorylation as a novel mitochondrial therapeutic strategy for IR injury that could confer protection by preventing the ischemic-induced dephosphorylation of ANT (Feng et al., 2008). The coordinated expression of two genomes, nuclear and mitochondrial, regulates the biogenesis of OxPhos (Garesse and Vallejo, 2001). One report suggested that sevoflurane induced delayed conditioning by activating nuclear factor-κB (NF-κB) (Qiao et al., 2013), an inducible transcription factor produced in response to ROS and RNS, and that this modulation could help control the transcription of DNA and cellular responses to stress stimuli against myocardial injury by limiting apoptosis.

METABOLIC ROLE OF VA IN CARDIOPROTECTION

Mitochondria normally generate ATP by electron transport, H^+ pumping and OxPhos. But during ischemia the shortage in substrates and O_2 decreases OxPhos promoting the working of F_1F_0 ATPase in reverse. Reduced cellular ATP levels stimulate glycolysis causing lactic acidosis and a rise in intracellular Na^+ by activating the Na^+-H^+ exchanger (NHE). In turn this leads to an increase in intracellular Ca^{2+} by activating the sarcolemmal Na^+-Ca^{2+} exchanger (NCE) (Murphy and Steenbergen, 2008a), as shown in **Figure 2**. An alteration in mitochondrial membrane transport protein function, e.g., VDAC, can contribute to IR injury by impeding delivery of substrates required to carry out OxPhos. In an overview of VDAC functional regulation in cell death during cardiac IR, Das et al. (2012) suggested that less entry of nucleotides via VDAC during IR injury might protect cells by reducing the rate of ATP utilization. A decline in cell ATP utilization during ischemia is considered fundamental for cardioprotection in an IPC setting by improving ATP availability during reperfusion (Murry et al., 1986). Thus, a decrease in ATP production and reduced ATP entry into mitochondria through VDAC could lead to reduced ATP consumption by F_1F_0-ATPase, and to reduced glycolysis and lactic acidosis, which ultimately could lead to decreased cytosolic Ca^{2+} loading via cation exchangers (Murphy and Steenbergen, 2008a). A reduction in glycolysis also leads to decreased cytosolic H^+ and less H^+ entry into mitochondria.

The studies above indicate that alterations in mitochondrial membrane proteins play crucial roles in indirectly modulating mitochondrial Ca^{2+} overload and excess ROS emission. Indeed, modulation of VDAC function by VA, directly or indirectly, as reported by Jamnicki-Abegg et al. (2005), Raphael et al. (2006), and Zhu et al. (2010), may reduce the vicious feed-forward cycle of Ca^{2+} overload and ROS emission that culminates in cell demise. Given the numerous roles of VDAC in transfer of anion/cation and other metabolites, there could be additional mechanisms involving mitochondrial membrane proteins-induced cardioprotection and any role of VA in these complex mechanisms have yet to be explored.

EFFECTS OF VA ON SIGNAL TRANSDUCTION PATHWAYS DURING IR INJURY

Exposure to VA preceding IR leads to activation of several signaling cascades that involve protein kinases and "small" transient ROS/RNS, including $NO^{\cdot}$. These signaling molecules eventually converge on mitochondria to provide protection (Zaugg et al., 2003b; Walters et al., 2012). Marinovic et al. (2006) provided evidence in support of a dual role mK_{ATP} channels in VA protection, i.e., as a trigger to initiate the signaling cascade and as an effector responsible for the cardioprotective memory. APC with sevoflurane was reported to improve vascular and mechanical function by increasing $NO^{\cdot}$ release that was blocked by an mK_{ATP} channel inhibitor (Novalija et al., 1999). In an *in vivo* rat model, Ludwig et al. (2004b) suggested that APC is mediated by opening of mK_{ATP} channels and the subsequent generation of transient ROS, which activates protein kinases. In another study, Pravdic et al. (2009) inferred that APC is mediated by a PKC-δ-induced delay of mPTP opening. Lastly, another study showed that APC protected the mouse heart against reperfusion injury by preventing mPTP opening in an eNOS dependent manner, with $NO^{\bullet}$ acting as both the trigger and the mediator of cardioprotection (Ge et al., 2010).

The G protein coupled receptor 30 (GPR 30) agonist G1 improved cardiac function, reduced infarct size, and inhibited mPTP opening by activating ERK signaling in the isolated mouse hearts after IR (Bopassa et al., 2010). The pro-survival

kinases ERK1/2 and PI3K/Akt also appear to contribute to VA mediated cardioprotection (Raphael et al., 2005; Wang et al., 2006b). Heat shock protein 90 (HSP 90), a cytoprotective protein, facilitated the translocation of PKC-ε after IR, and increased phosphorylation of mitochondrial aldehyde dehydrogenase 2 (ALDH2) (Budas et al., 2010). ALDH2 is known to detoxify 4-hydroxynonenal (HNE), a cytotoxic end product of lipid peroxides following oxidative stress, by oxidizing the aldehyde group (Camara et al., 2010). In this case, increased ALDH2 activity resulted in reduced cardiac injury in an animal model of myocardial infarction (Budas et al., 2010). A recent study showed that isoflurane-induced APC alleviated hypoxia-reoxygenation injury in conjunction with PKC-δ mediated activation of mitochondrial ALDH2 (Lang et al., 2013). Bouwman et al. (2006) reported that activation of PKC-δ by sevoflurane increased sarcolemmal NCE mediated myocardial Ca^{2+} influx, which may be a trigger of cardioprotective signaling events during APC. Desflurane preconditioning was reported to activate BK_{Ca} channels through protein kinase A (PKA) (Redel et al., 2008). Exposure to isoflurane during early reperfusion induced cardioprotection associated with increased expression of the anti-apoptotic Bcl-2, a modulator of mPTP (Wang et al., 2006a). Isoflurane also protected hearts from IR injury, possibly by preventing excess ROS generation and mPTP opening that in turn inhibited the activation of caspase-3 (Wu et al., 2014). Although the signaling pathways are very complex and incompletely resolved, it is likely that VA modulate other known and unknown mitochondrial channel/transporters involved in IR injury; but this remains to be tested.

OTHER POTENTIAL MITOCHONDRIAL TARGETS OF VA DURING IR INJURY

POST-TRANSLATIONAL MODIFICATIONS

Beneficial post-translational modifications (PTMs) of mitochondrial proteins have been proposed to modulate cardioprotection (Foster et al., 2009; Pagliaro et al., 2011; Porter et al., 2012). A modification of mitochondrial protein by O-glycosylation with O-linked-β-N-acetyl glucosamine (O-GlcNAc) was suggested to occur with IPC as assessed by improved cardiac myocyte survival due to attenuated $\Delta\Psi_m$ (Jones et al., 2008). In a recent study (Champattanachai et al., 2008) it was reported that the protection by increased O-GlcNAc during injury of neonatal rat ventricular myocytes was mediated by enhanced mitochondrial Bcl-2 translocation. *In vivo* and *ex vivo* studies with isoflurane preconditioning in mice demonstrated increased O-glycosylation of cardiac mitochondrial VDAC associated with resistance to IR stress (Hirose et al., 2011).

VA-mediated PTM (mostly phosphorylation) of mitochondrial proteins involved in bioenergetics and electron transport complexes are implicated in the role of PTMs in regulating mitochondrial function that confers cardioprotection (Arrell et al., 2006; Kalenka et al., 2006; Wong et al., 2010). However, additional studies are needed to validate the functional effects of these changes during the various conditioning periods against IR injury. Signaling RNS can also induce beneficial, reversible PTMs. Specifically, S-nitrosylation of some mitochondrial proteins may lead to cardioprotection during IPC and IPoC (Tullio et al., 2013). As noted before, complex I dysfunction resulting from oxidative damage is an important factor in the pathogenesis of IR injury (Murray et al., 2003). Therefore, another possible mechanism of cardioprotection is modulation of complex I protein by $NO^{\cdot}$-induced S-nitrosation leading to beneficial modulation of bioenergetics and redox signaling (Burwell et al., 2006). Complex IV is another target of $NO^{\cdot}$ where it competes with O_2 at its binding site (Brookes et al., 2001); Similarly, VA were also reported to modulate complex IV activity and to alter its function (Casanovas et al., 1983; Szabo and Zoratti, 1993); however, whether this was through $NO^{\cdot}$ was not evident. As noted earlier, in our recent study (Agarwal et al., 2014), we did not observe an effect of isoflurane on complex IV function. This discrepancy in VA modulation of mitochondrial function as a cardioprotective strategy further supports the complexity of VA interaction with mitochondrial proteins.

Recently, changes in the mitochondrial proteome during APC were assessed by a proteomic mass spectral approach (Bienengraeber et al., 2013). An ^{18}O-labeling method was applied to relatively compare cardiac mitochondrial samples from control and isoflurane exposed rats before and after IR. It was found that the activities of ATP synthase, a complex I subunit, citrate synthase, and isocitrate dehydrogenase were increased after APC compared to IR only based on phosphorylation of the proteins (Bienengraeber et al., 2013). Since, those modulated proteins directly belong to the OxPhos system, these observations further confirm the role of VA in altering mitochondrial bioenergetics/metabolism.

MicroRNAs

miRNAs are endogenous, small non-coding, single stranded RNAs (ssRNAs, ∼22 nucleotides) that are involved in transcriptional and post-transcriptional regulation of gene expression (Chen and Rajewsky, 2007). Several recent reports suggest miRNAs are novel therapeutic biomarkers for IR injury (Cheng et al., 2010a), but their potential application in myocardial protection against IR is not known. The up- or down-regulation of miRNAs have been reported to occur during IR; in particular, protective effects of miRNAs with their target genes were identified that reduced cardiac cell apoptosis during pre- and post- conditioning against cardiac IR injury (Dong et al., 2009; Cheng et al., 2010b; He et al., 2011). One study reported that upregulation of miRNAs was involved in delayed preconditioning, in which miRNAs appeared to upregulate proteins (eNOS, HSP70) involved in delayed preconditioning after IPC (Yin et al., 2009). The role of miRNAs in APC or as a direct target of VA has not been reported. However, according to a recent preliminary report by Olson et al. (2013), *in vitro* application of isoflurane caused upregulation of miR-21 and conferred cardioprotection, while knockdown of miR-21 attenuated cardioprotection. Moreover, in that study attenuation of APC during acute hyperglycemia was also linked to regulation of miR-21; i.e., overexpression of miR-21 in cells exposed to high glucose restored APC via the Akt/GSK3β link and increased cell survival.

MITOCHONDRIAL DNA

Mitochondria have their own genome that comprises only a small portion of the total eukaryotic cell genome. The mitochondrial

DNA (mDNA) encodes 13 mitochondrial proteins and the mitochondrial rRNAs and tRNAs needed for translation (Kirby and Thorburn, 2008). Unlike the nuclear DNA, mDNA is not protected by histones and is therefore susceptible to damage by oxidative stress (Camara et al., 2010). A decrease in mDNA is a biological maker of myocardial damage, as in cardiac hypertrophy, that progresses to heart failure (Karamanlidis et al., 2011). A recent study (Muravyeva et al., 2014) examined if mDNA modulates APC and cardiac susceptibility to IR injury by using two strains of diabetic rats following exposure to isoflurane. The study proposed that differences in the mitochondrial genome modulate isoflurane-induced generation of ROS that translates into a differential susceptibility to APC; this suggested a potentially important role of mDNA in regulating cardioprotection in APC via modulation of ROS production.

CONCLUSIONS AND FUTURE DIRECTIONS

Improvement in the clinical management of ischemic heart disease remains elusive despite the discovery of many molecular and cellular mechanisms that may be valuable targets to treat against IR injury. The importance of mitochondrial bioenergetics and function in contributing not only to cardiac injury but also to reducing cardiac injury is now well recognized. But there remains a lack of clear understanding of the mitochondrial-cytosolic mechanisms that might lead to more targeted intervention. Hence, we need to identify new targets that could uncover the mechanisms of dysfunction associated with IR injury. With a better understanding of mitochondrial targets as hubs for controlling metabolism and cellular redox signaling pathways that elicit protection, we could better develop novel therapeutic drugs for clinical trials to protect against IR injury. Because VA are lipophilic agents with multi-targeted actions that, together, confer cardioprotection, they give us valuable clues into which potential sites to investigate; these clues may be especially useful to selectively and reversibly target mitochondria to reduce IR injury. Unfortunately, as summarized in this review, there is contradictory evidence with respect to the potentially large number of pathways by which VA might protect the heart. Nonetheless, there are molecules with characteristics of a VA but without anesthetic properties. These could be developed as cardioprotective drugs while obviating the need for inducing anesthesia.

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Far red/near infrared light-induced protection against cardiac ischemia and reperfusion injury remains intact under diabetic conditions and is independent of nitric oxide synthase

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Far red/near-infrared light (NIR) promotes a wide range of biological effects including tissue protection but whether and how NIR is capable of acutely protecting myocardium against ischemia and reperfusion injury *in vivo* is not fully elucidated. Our previous work indicates that NIR exposure immediately before and during early reperfusion protects the myocardium against infarction through mechanisms that are nitric oxide (NO)-dependent. Here we tested the hypothesis that NIR elicits protection in a diabetic mouse model where other cardioprotective interventions such as pre- and postconditioning fail, and that the protection is independent of nitric oxide synthase (NOS). NIR reduced infarct size dose dependently. Importantly, NIR-induced protection was preserved in a diabetic mouse model (db/db) and during acute hyperglycemia, as well as in endothelial $NOS^{-/-}$ mice and in wild type mice treated with NOS inhibitor L-NAME. In *in vitro* experiments NIR light liberates NO from nitrosyl hemoglobin (HbNO) and nitrosyl myoglobin (MbNO) in a wavelength-(660-830 nm) and dose-dependent manner. Irradiation at 660 nm yields the highest release of NO, while at longer wavelengths a dramatic decrease of NO release can be observed. Similar wavelength dependence was observed for the protection of mice against cardiac ischemia and reperfusion injury *in vivo*. NIR-induced NO release from deoxymyoglobin in the presence of nitrite mildly inhibits respiration of isolated mitochondria after hypoxia. In summary, NIR applied during reperfusion protects the myocardium against infarction in an NO-dependent, but NOS-independent mechanisms, whereby mitochondria may be a target of NO released by NIR, leading to reduced reactive oxygen species generation during reperfusion. This unique mechanism preserves protection even during diabetes where other protective strategies fail.

Keywords: near infrared light, nitrite reductase, ischemia and reperfusion injury, myoglobin, cardioprotection

INTRODUCTION

Restoration of blood flow to a region of previously ischemic myocardium (reperfusion) is a critical life-saving intervention against tissue necrosis, but reperfusion itself also results in significant damage to the myocardium. Many therapeutic strategies such as ischemic and volatile anesthetic pre- and postconditioning have been developed and are effective in healthy animal models but few have translated successfully to patients (Ludman et al., 2010). A major reason for the resistance to cardioprotection against infarction by physical or pharmacological stimuli is the advanced age and/or presence of comorbidities such as diabetes in patients. For example, endothelial dysfunction appears to contribute to the lack of protection by ischemic or anesthetic postconditioing in diabetes (Raphael et al., 2010; Przyklenk et al., 2011). Activation of endothelial nitric oxide synthase (eNOS) and pro-survival signaling pathways, together with alteration of mitochondrial bioenergetics, contribute to the mechanisms of various cardioprotective strategies against ischemia and reperfusion injury (Tsang et al., 2004; Mio et al., 2009; Ge et al., 2010). Although nitric oxide synthases (NOS) produce a large part of endogenous nitric oxide (NO), there is considerable interest in NOS-independent generation of NO *in vivo*, particularly during hypoxia or anoxia, where low oxygen tensions limit NOS activity (Godecke, 2006; Hendgen-Cotta et al., 2008, 2010). Interventions that can increase NO bioavailability have significant therapeutic potential. Under hypoxic conditions, heme-containing proteins such as myoglobin (Mb) and hemoglobin (Hb) exhibit nitrite reductase activity which results in an increase in NO bound to the heme iron of Mb and Hb (Gladwin et al., 2006; Hendgen-Cotta et al., 2008). We have recently found that far red/near infrared

light (NIR) both in purified systems and in myocardium can release NO from nitrosyl hemes (Lohr et al., 2009). Further, NIR protected cardiomyocytes and the rabbit heart from hypoxia and reoxygenation injury in a NO-dependent manner, reversible by NO scavenger cPTIO, and enhanced the protective effect of nitrite against ischemia and reperfusion injury of the rabbit heart (Lohr et al., 2009; Zhang et al., 2009).

NIR modulates biochemical systems by activating light-sensitive proteins harboring NIR-sensitive chromophores (Karu, 1999; Desmet et al., 2006). Previous studies suggested that NIR promotes cell survival during physiologic stress (Eells et al., 2003; Liang et al., 2008; Zhang et al., 2009). Repeated photostimulation of the myocardium has been demonstrated to be beneficial against long-term reperfusion injury in the rat and dog (Oron et al., 2001). For example, low-energy infrared (803 nm) laser irradiation delivered to the epicardium was shown to reduce scar formation and myocardial infarct size several weeks after prolonged coronary artery occlusion in dogs and rats (Oron et al., 2001). Aside from the heart, the beneficial effects of NIR light treatment have been studied in particular in a model of traumatic brain injury as well as in wound healing (Ankri et al., 2010; Naeser et al., 2011). NIR light treatment also improved the collateral blood vessel grow in a mouse model (tight skin mouse) of scleroderma (Zaidi et al., 2013). Frequently, the beneficial effects of NIR treatment have been associated with the stimulation of mitochondrial metabolism, particularly at the level of cytochrome c oxidase, complex IV of the electron transport chain (Karu, 2008). However, in a model of cardiac ischemia and reperfusion injury it is difficult to perceive how acceleration of cytochrome c oxidase at the time of reperfusion conveys protection to the heart. Rather, a mild reversible inhibition of the electron transport chain has been shown to reduce reactive oxygen species production during reperfusion, thereby increasing cardiomyocyte survival (Burwell et al., 2009). NO inhibits electron transport through competitive binding at complex IV and S-nitrosation at complex I (Piantadosi, 2012; Chouchani et al., 2013). Thus, we tested the hypothesis that brief exposure to NIR light at the time of reperfusion protects the heart in a wave length-dependent manner; and that this wave length dependence is paralleled by the release of NO from nitrosyl-heme proteins. We also examined whether NIR induced protection is maintained in a mouse model of acute hyperglycemia and diabetes (db/db) where protection by volatile anesthetics fail.

MATERIALS AND METHODS

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the Medical College of Wisconsin. Furthermore, all conformed to the *Guiding Principles in the Care and Use of Animals* of the American Physiologic Society and were in accordance with the *Guide for the Care and Use of Laboratory Animals*.

MYOCARDIAL ISCHEMIA AND REPERFUSION INJURY IN MICE

A murine model of myocardial ischemia and reperfusion injury was used as previously described (Ge et al., 2010). C57Bl/6 (wild type) mice, as well as eNOS$^{-/-}$ and diabetic db/db mice were used for these experiments. Glucose (2 g/kg) was administered intraperitoneal 10 min before ischemia to produce hyperglycemia. Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (100 mg/kg) and ventilated with room air supplemented with 100 % oxygen at a rate of 100 breaths/min with a tidal volume of approximately 0.25 ml using a rodent ventilator (Harvard Apparatus, South Natick, MA). Body temperature was maintained between 36.8 °C and 37.5 °C. Myocardial ischemia was produced by occluding the left coronary artery (LAD) for 30 min, and reperfusion was initiated by loosening the suture and continued for 3 h.

EXPERIMENTAL PROTOCOL

Mice were randomly assigned to receive no irradiation (control) or NIR irradiation applied to the epicardial surface (670 nm, 170 mW/cm^2) with an LED array (NIR Products LLC, Milwaukee, WI) for 1 min before and through the first 4 min of reperfusion (energy-density equivalent to 51 J/cm^2). Separate experiments were performed to evaluate the energy- and wavelength-dependence of NIR-mediated cardioprotection by varying the array (670, 740, 830 nm) and the output of the device.

C57BL/6, eNOS$^{-/-}$ mice and db/db mice were used to explore the dependence of NIR-mediated cardioprotection on eNOS and its efficacy in a diabetic animal.Acute hyperglycemia was induced by administration of D-glucose (2 g/kg) in C57BL/6 mice 10 min before ischemia. Mannitol (1.82 g/kg) was used for osmotic control in preliminary experiments in C57BL/6 mice both with and without NIR treatment and did not exhibit any significant effect compared to mice that did not receive mannitol. Pharmacological inhibition of eNOS was used to complement the experiments in eNOS$^{-/-}$ mice and thus, C57BL/6 mice received 1mg/kg, i.v. of the non-selective NOS inhibitor L-NAME prior to LAD occlusion and reperfusion.

DETERMINATION OF MYOCARDIAL INFARCT SIZE

For infarct size measurements, the heart was first stained by cannulation of the aorta with a 1 % solution of 2,3,5-triphenytetrazolium chloride. Then the suture previously placed around the left descending coronary artery was retied and diluted phthalo blue dye was injected through the same cannula. As a result of these procedures, the non-ischemic portion of the left ventricle was stained dark blue. Viable myocardium within the area at risk was stained bright red, and infarcted tissue was light yellow. The heart was then excised and ventricles were cut into 4–5 uniform transverse slices of 2 mm thickness using a mouse heart matrix. Slices were then analyzed by planimetry.

NITROSYL HEMOGLOBIN (HbNO) AND NITROSYL MYOGLOBIN (MbNO) PREPARATION

Oxyhemoglobin purified from human blood according to a published procedure (Rossi-Fanelli et al., 1961) was deoxygenated, or solution of metmyoglobin (from horse skeletal muscle, Sigma) was reduced in an anaerobic chamber with $Na_2S_2O_4$ in phosphate buffered saline (PBS, pH 7.4). Then the heme proteins were nitrosylated by addition of equivalent concentration of highly concentrated PROLI NONOate (Cayman Chemicals, Ann

Arbor, Mi) dissolved in 0.1 N NaOH. The process was spectrophotometrically followed. Solutions were made daily, and used immediately.

NO-DEPENDENT CHEMILUMINESCENCE ANALYSIS

A Sievers 280i Nitric Oxide Analyzer (General Electric, Boulder, CO) was used to detect NO evolved from nitrosyl species as a consequence of NIR irradiation. HbNO or MbNO (3 ml of 10 μM) was placed into the purge vessel of the analyzer, and externally irradiated at various powers and wavelengths for 1 min. Detector response for NO liberated from known amounts of PROLI NONOate injected into PBS pH 7.4 was used as a basis of quantification.

MEASUREMENT OF OXYGEN CONSUMPTION IN ISOLATED MITOCHONDRIA

Rat heart mitochondria were isolated by differential centrifugation as previously reported (Pravdic et al., 2010). Mitochondrial oxygen consumption was measured with a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, UK) at 30 °C in respiration buffer containing mitochondria at a final concentration of 1 mg protein/mL. The mitochondrial respiration buffer was composed of 130 mM KCl, 5 mM KH_2PO_4, 20 mM MOPS, 2.5 mM EGTA, 1 mM $Na_4P_2O_7$, and 0.1 % BSA, at pH 7.4. State 2 respiration was initiated with 5 mM pyruvate and 5 mM malate as substrates. The adenosine diphosphate (ADP)-stimulated oxygen consumption (state 3 respiration) was measured in the presence of 250 μM ADP. After hypoxia was reached mitochondria were incubated with deoxymyoglobin (40 μM; prepared from myoglobin with sodium dithionite as reducing agent) and sodium nitrite (20 μM) for 1 min and then exposed directly to NIR (170 mW/cm^2) for another min. After that the chamber was opened to allow reoxygenation. A faster rate of reoxygenation of the chamber indicated an inhibition of respiration.

STATISTICAL ANALYSIS

Statistical analysis of data within and between groups was performed with analysis of variance (ANOVA) for repeated measures followed by the Student-Newman-Keuls test. Changes were considered statistically significant when $P < 0.05$. All data are expressed as mean ± standard deviation (SD) unless otherwise indicated.

RESULTS

The mouse was chosen as model to determine the efficacy of NIR-mediated protection against cardiac ischemia and reperfusion injury in order to expand our findings on NIR-induced protection in rabbits and due to the advantage of the large availability of genetically engineered animals. Exposure to NIR for the last min of occlusion and first 4 min of reperfusion significantly ($P < 0.05$) reduced infarct size at the highest chosen irradiance (170 mW/cm^2, corresponding to 51 J/cm^2) compared to control experiments without NIR exposure (31 ± 7 vs. 51 ± 4 % of left ventricular area of risk, **Figure 1**). The effect of NIR on infarct size was energy dependent. Myocardial infarct size was 59 ± 5 and 39 ± 6 %, at an irradiance of 10 and 27 mW/cm^2, corresponding to 3 and 8.1 J/cm^2 respectively. Thus, the threshold of cardioprotection appeared to occur at an irradiance level of 30 mW/cm^2.

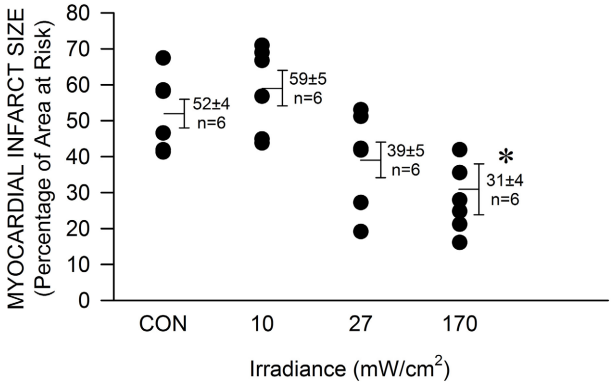

FIGURE 1 | The NIR-mediated cardioprotection is irradiance-dependent. The exposed mouse heart was irradiated at 670 nm with various irradiances for 1 min during ischemia and 4 min during reperfusion. The lamp was placed 5 mm above the heart. At 170 mW/cm^2 a significant decrease in infarct size was observed. Values are means ± SD, $^*p < 0.05$ compared to untreated control (CON).

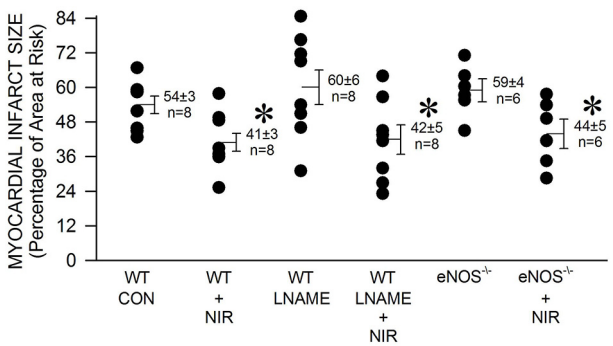

FIGURE 2 | The NIR-mediated cardioprotection is NOS-independent. The exposed heart of wild type and eNOS$^{-/-}$ mice was irradiated at 670 nm with 170 mW/cm^2 power for 1 min during ischemia, and 4 min during reperfusion. Mice treated with L-NAME (10 mg/kg, administered 10 min prior occlusion) were also examined. Irradiation was equally protective in the case of control, L-NAME-treated, and eNOS$^{-/-}$ mice. Values are means ± SD, $^*p < 0.05$ when compared to untreated control (CON).

Importantly, no increase in epicardial surface temperature upon exposure to NIR was observed.

We then tested whether NOS is involved in the mechanism of NIR-induced protection. Pretreatment with the non-selective NOS inhibitor L-NAME had no effect alone (60 ± 6 % infarct size of area at risk), nor did it inhibit NIR-mediated reduction in infarct size (42 ± 5 %). Similarly, eNOS$^{-/-}$ mice were also protected against myocardial ischemia and reperfusion injury by NIR treatment (44 ± 5 % compared to 59 ± 4 % without treatment). An irradiance of 170 mW/cm^2 was applied in all cases. These data suggest that NIR-mediated cardioprotection is independent of the activity of NOS (**Figure 2**).

We recently reported (Lohr et al., 2009) that NIR light has the capacity to liberate NO from nitrosylated hemoglobin (HbNO) and myoglobin (MbNO). Here we examined the wavelength dependence of NO release and protection. A solution of

HbNO or MbNO (10 μM) was placed into the purge vessel of a chemiluminescence-based NO analyzer and subjected to irradiation for 1 min. We observed 2–3 times more NO released at 670 nm compared to 740 and 830 nm at 10 mW/cm^2 irradiance (**Figure 3A**). As NIR does not have to penetrate tissue in these experiments, less irradiance compared to what is required for protection of the *in vivo* heart is needed. There was no significant difference between NO liberation from HbNO and MbNO. A similar trend was found with the wavelength dependence of NIR-induced reduction of infarct size. In contrast to 670 nm no significant protection was observed at 740 and 830 nm (**Figure 3B**).

Mitochondria are a potential therapeutic target of NO produced at the time of reoxygenation (Chouchani et al., 2013). Therefore, experiments were designed to establish the net outcome of NIR-enhanced nitrite reductase activity on mitochondrial respiration after hypoxia. We measured the reoxygenation rate of a mitochondrial suspension after hypoxia in the presence of deoxymyoglobin and nitrite, with and without NIR (660 nm, 50 mW/cm^2) (**Figure 4**). While Mb and nitrite induce inhibition of mitochondrial respiration alone (Shiva et al., 2007a,b; Hendgen-Cotta et al., 2008), we hypothesized that light enhances this inhibition through its action on MbNO formed as a consequence of nitrite reductase activity of heme. We found that NIR, while alone had no considerable effect, could potentiate the inhibition caused by Mb and nitrite at lower nitrite doses. It triggered a significantly faster reoxygenation and thereby a decrease in respiration rate in the presence of deoxymyoglobin and nitrite than solely deoxymyoglobin and nitrite would induce. A partial compensatory effect of NO bound to and released from complex IV cannot be excluded, however, in the investigated *in vitro* system Mb was present in wide excess over cyt c oxidase (0.64 mg/ml Mb vs. 1 mg/ml total mitochondrial protein), and NIR was switched off at the time of reoxygenation. Thus, NO released from Mb might partially bind to cytochrome c oxidase or mediate S-nitrosation of complex I at the beginning of reoxygenation, thereby accelerating reoxygenation and inhibiting respiration. The observed effect is relevant since a mild reversible inhibition of the mitochondrial electron transport chain during cardiac reperfusion has been shown to reduce reactive oxygen

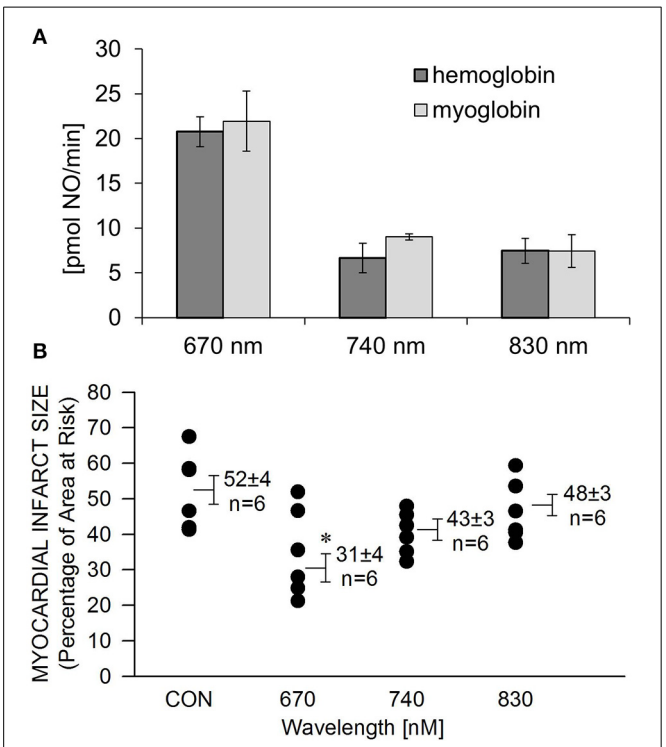

FIGURE 3 | Wavelength-dependence of NIR-induced NO release is in accordance with wavelength dependence of cardioprotection. (A) A solution of HbNO or MbNO (10 μM in PBS, pH 7.4) was irradiated at various wavelengths with 10 mW/cm^2 power for 1 min in the chamber of the NO analyzer. Formation of NO was on-line detected with ozone-based chemiluminescence. Maximum NO liberation was achieved at 660 nm irradiation with both heme nitrosyl compounds. Values are means ± SE, $n = 3$. **(B)** The exposed heart was irradiated at various wavelengths at170 mW/cm^2 irradiance for 1 min during ischemia, and 4 min during reperfusion. Irradiation at 670 nm was the most cardioprotective. Values are means ± SD, $^*p < 0.05$ when compared to untreated control (CON).

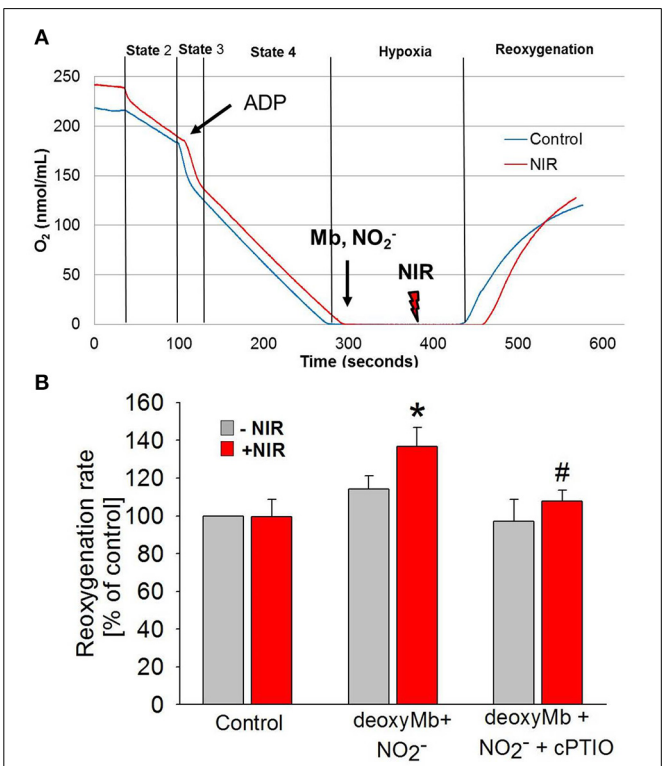

FIGURE 4 | NIR slows mitochondrial respiration in the presence of deoxymyoglobin and nitrite during reoxygenation. (A) Oxygen consumption of purified cardiac mitochondria was monitored with a Clarke-type oxygen electrode. At the beginning of ischemia, deoxyMb and sodium nitrite were added. During the second minute of ischemia before reoxygenation, mitochondria were exposed to NIR at 670 nm with 50 mW/cm^2 irradiance. **(B)** NIR caused an NO-dependent increase of reoxygenation rate (corresponding to a decrease of respiration) in the presence of deoxyMb and nitrite. The effect was reversed with NO scavenger CPTIO. Values are means ± SD, $n = 6$, $^*p < 0.05$ when compared to control, $^\#p < 0.05$ when compared to NIR treated mitochondria in the absence of CPTIO.

species production. In the presence of NO scavenger cPTIO (10 μM), the NIR effect was reversed.

In diabetes, endothelial dysfunction, including defective NOS, is considered one of the causes for the failure of protective strategies such as ischemic or anesthetic pre- and postconditioning to reduce cardiac ischemia and reperfusion injury. Therefore, a NOS-independent mechanism of NO generation may allow NIR to reduce ischemia and reperfusion injury in the hyperglycemic or diabetic heart. Indeed, a similar degree of NIR-induced cardioprotection was observed in mice that were exposed to acute hyperglycemia (39 ± 4 % myocardial infarct size of area at risk vs. 52 ± 2 % without NIR) and in the diabetic db/db mouse (43 ± 4 vs. 61 ± 3 %) compared to the wild type mice (41 ± 3 % vs. 56 ± 3 %).

DISCUSSION

The current results demonstrate that a brief exposure to NIR immediately before and during early reperfusion protects the myocardium against infarction in an NOS-independent mechanism. Mitochondria are one potential therapeutic target of NIR-induced release of NO but other targets such as NO-sensitive guanylyl cyclase require further investigation. Importantly, NIR protects the hyperglycemic and diabetic heart. The absence of such protections has been one of the major hurdles in the implementation of pharmacological pre- and particularly postconditioning into the clinical setting.

In the nineteen nineties in Russia patients with coronary heart disease with prior myocardial infarction were exposed repeatedly to NIR by low-level laser therapy (LLLT) applied to the area of the heart on the skin. Lipid peroxidation was significantly reduced after NIR but little is known on whether cardiac function improved (Zubkova et al., 1993; Sorokina et al., 1997). In subsequent studies NIR was applied after chronic myocardial infarction in rat and dog models. NIR (803 nm, 6 mW/cm^2 at the surface of the myocardium for 3 min, at 4-6 different locations) was applied twice, 15 min and 3 days after myocardial infarction, through the open chest directly onto the myocardium in dogs, and through the intercostal muscles in rats. Both mortality and infarct size were significantly reduced compare to untreated animals (Oron et al., 2001). Irradiation with NIR after myocardial infarction in rats resulted in a significant improved mitochondrial bioenergetics, and an increase in an inducible heat shock protein (HSP70), vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS) expression (Yaakobi et al., 2001). This was paralleled by a significant elevation in angiogenesis (Tuby et al., 2006). More recently, increased angiogenesis and collateralization upon NIR exposure (670 nm, 50 mW/cm^2, 10 min per day for 14 days) with LED have also been reported in the ischemic hind limb of mice and rabbits (Lohr et al., 2013). In a mouse model for systemic sclerosis, an autoimmune connective tissue disorder characterized by oxidative stress, impaired vascular function, and attenuated angiogenesis, NIR stimulated angiogenesis by increasing angiomotin and decreasing angiostatin expression in the ischemic hind limb (Zaidi et al., 2013).

In the present study we found that myocardial infarction can be prevented from occurring, or at least reduced by a one-time NIR treatment right at the time of reoxygenation. It appears highly unlikely that NIR-induced reductions in infarct size were attributed to increased collateral perfusion as NIR-induced angiogenesis typically occurs as a result of sustained stimulation over several days. Therefore, the underlying mechanism of protection is likely different. Under hypoxic conditions heme-containing proteins such as myoglobin (Mb) and hemoglobin (Hb) exhibit nitrite reductase activity which results in an increase in NO liberation (Gladwin et al., 2006; Hendgen-Cotta et al., 2008). The NO formed may subsequently react with available deoxyHb or deoxyMb to yield iron-nitrosyl Hb (HbNO) or iron-nitrosyl Mb (MbNO). Thus, HbNO and MbNO may represent a significant storage pool of NO in the heart. Here we have demonstrated both for purified hemoglobin and myoglobin that NIR can decay nitrosyl heme and release NO in a wavelength-dependent manner. Importantly, the highest NO release was recorded at 670 nm where protection against ischemia and reperfusion injury was present (**Figure 3**). This further suggests a distinct mechanism from the previously reported protection through repeated NIR treatment in the permanently ligated heart where longer wavelengths were equally protective. We previously reported in the ischemic rabbit heart, after infusion of sodium nitrite, a large increase in nitrosyl heme formation as measured by electro paramagnetic resonance spectroscopy (EPR). The MbNO signal was reduced in the ischemic zone by NIR treatment suggesting dissociation of the heme-NO bond upon irradiation (Lohr et al., 2009).

Frequently, the beneficial effects of NIR treatment have been associated with the stimulation of mitochondrial metabolism, particularly at the level of cytochrome c oxidase, complex IV of the electron transport chain and concomitant enhancement of ATP synthesis (Karu, 2008). NIR may directly affect cytochrome c oxidase activity through one of its redox active metal centers. In addition, it has been suggested that NIR exerts its action on cytochrome c oxidase by a mechanism via NO release. The activated cytochrome c oxidase may not only cause changes in electron transport chain activity, including ROS generation, but released NO is available for other biological processes such as vasodilation and gene expression. However, compared to potential NO release from HbNO or MbNO the relative amounts of NO in the case of cyt c oxidase is limited (Osipov et al., 2007). Further, it is difficult to perceive how acceleration of cytochrome c oxidase at the time of reperfusion conveys protection to the heart. Rather, a mild reversible inhibition of the electron transport chain has been shown to reduce reactive oxygen species production during reperfusion and increase cardiomyocyte survival (Burwell et al., 2009). This was confirmed in ischemic isolated mitochondria where, in the presence of deoxmyoglobin and sodium nitrite, a decrease in respiration was detected upon reoxygenation of mitochondria after application of NIR (**Figure 4**). NO signaling may lead to S-nitrosation of a cysteine residue in complex I that has been implicated in protection against cardiac ischemia and reperfusion injury (Cochain et al., 2013). Reversible S-nitrosation of complex I slows the reactivation of mitochondria during the crucial first minutes of the reperfusion of ischemic tissue, thereby decreasing ROS production, oxidative damage and tissue necrosis.

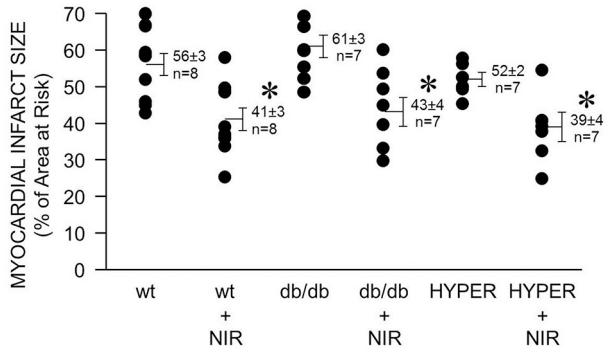

FIGURE 5 | NIR is cardioprotective in the presence of diabetes or hyperglycemia. The exposed heart of wild type, db/db, and hyperglycemic mice was irradiated at 670 nm with 170 mW/cm^2 irradiance for 1 min during ischemia, and 4 min during reperfusion. Light exposure resulted in a similarly effective decrease of infarct size in all three cases. Values are means ± SD, $^*p < 0.05$ when compared to untreated wild type.

Due to the reasonably high tissue penetration paralleled by limited potential of tissue damage NIR is attractive for the use in ischemic heart disease. While the required light power needs to be verified for human cardiac use, by comparing animal studies through various species and experimental settings an irradiance of 10–100 mW/cm^2 for 2–10 min seems a reasonable starting point to achieve beneficial effects of NIR. A higher irradiance may be required for acute prevention of ischemia and reperfusion injury at the time of reperfusion. NIR treatment of the heart may be protective on patients after acute myocardial infarction or on ischemic heart conditions that are not accessible to current revascularization procedures. NIR could be particularly useful in the presence of comorbidities such as diabetes. Diabetes is an independent predictor of increased cardiovascular risk and myocardial infarct size is directly related to increases in blood glucose concentration in animals with or without diabetes (Van Der Horst et al., 2007). The mechanism of light-induced release of NO from iron-nitrosylated heme protein is likely to be maintained during diabetes and thus NIR may be protective from ischemia and reperfusion injury where other strategies such as ischemic and pharmacologic pre- and postconditioning fail (Kersten et al., 1998; Przyklenk et al., 2011). Indeed, we found that under acute hyperglycemia or in a mouse model of type 2 diabetes (db/db mouse) NIR exposure of the mouse heart at the time of reperfusion reduces infarct size significantly (**Figure 5**).

While there is substantial clinical promise for the use of NIR in heart disease several hurdles need to be considered and overcome. The technical challenge related to the application of NIR to the heart due to limited penetration through muscle and bone is an important issue to consider. Obvious scenarios would be application of light where needed during cardiac surgery such as coronary artery bypass that carry a significant increased risk of myocardial infarction, or heart transplantation. It might also be possible to apply NIR during a balloon angiography, using a catheter bearing fiber optic through which the light can be delivered to the infarcted area. In addition, authors' unpublished data on dogs demonstrate the feasibility of a transesophageal approach to the heart with a flexible fiber optic NIR probe. The probe in the esophagus or stomach (when advanced) is immediately adjacent to the left atrium and the inferior and posterior walls of the left ventricle. Thus, the anterior wall that is frequently affected by myocardial infarction may be as much as 6 cm away from the probe. Still, it may not be necessary for NIR light to penetrate the area at risk directly. A remote effect of NIR, comparable to remote preconditioning, might still provide protection and lead to a reduction of infarct size. Signaling factors such as heat shock proteins or NO may mediate such effect.

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Blue LEDs get the Nobel Prize while Red LEDs are poised to save lives

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Ischemic heart disease is a major public health epidemic and a leading cause of morbidity and mortality worldwide (Hausenloy et al., 2012; Ferdinandy et al., 2014). Ischemic injury predisposes to myocardial infarction, heart failure, arrhythmias, and sudden cardiac death. Prompt restoration of oxygenated blood flow to the ischemic myocardium (i.e., reperfusion) is required for preventing irreversible cell damage and death. Unfortunately, restoration of blood flow, in itself, results in additional cardiac damage, known as reperfusion injury. Such oxidative damage, which is mediated by bursts of reactive oxygen species (ROS), is more severe when reperfusion therapy is delayed. Indeed, necrotic cell death as a consequence of ROS overproduction can paradoxically exacerbate the extent of myocardial infarction. Concomitantly, reperfusion-mediated cytosolic calcium overload and redox imbalance promote mechanoelectrical dysfunction and arrhythmias.

In recent years, mitochondria have emerged as central mediators of cell death and survival pathways (O'Rourke et al., 2005). On the one hand, opening of energy-dissipating mitochondrial channels that destabilize the mitochondrial membrane potential, such as the permeability transition pore (PTP) and the inner membrane anion channel (IMAC), result in myocardial infarction (Hausenloy et al., 2012) and arrhythmias (Akar et al., 2005), respectively. On the other hand, the seminal discovery of intrinsic cardioprotective pathways that stem from a mitochondrial origin has provided hope for combatting ischemia-reperfusion injury along with its pathological manifestations (impaired contractile recovery, arrhythmias, and myocardial infarction) (Murry et al., 1986). In particular, the proven efficacy of ischemic pre- and post-conditioning protocols in limiting the damage imposed by the index ischemic event has provided researchers with an effective tool for uncovering endogenous cardioprotective signaling pathways, with the promise of identifying molecular targets that can be manipulated pharmacologically (Ferdinandy et al., 2014).

Prominent amongst such targets are ATP-sensitive potassium channels in the mitochondrial membrane (mK_{ATP}) which are tightly regulated by PKC signaling. Although the molecular identity of these channels has eluded discovery for many years, recent work by the O'Rourke laboratory convincingly points to ROMK as a viable candidate (Foster et al., 2012). Nonetheless mK_{ATP} activation by diazoxide is cardioprotective against ischemia-reperfusion injury. Another key target is the PTP whose opening represents a terminal event that causes necrotic cell death. Indeed, Hausenloy and others have shown that PTP inhibition using cyclosporine-A (CsA) effectively limits the extent of myocardial infarction (Hausenloy et al., 2012). Whether CsA protects or exacerbates post-ischemic electrical dysfunction, however, remains a matter of debate. This issue may be complicated by PKC-dependent cross-talk between the PTP and mK_{ATP} channels which we recently examined (Xie et al., 2014). Moreover, post-ischemic arrhythmias can be suppressed by stabilizing the mitochondrial membrane potential using antagonists of the peripheral benzodiazepine receptor which modulates IMAC (Akar et al., 2005). The efficacy of this strategy in limiting infarct size, however, has not been systematically tested. Finally, volatile anesthetics have also been shown to reduce reperfusion injury likely by targeting mitochondrial pathways (Agarwal et al., 2014). Because pharmacological therapies for reperfusion injury have proven difficult, novel approaches for this epidemic are much needed.

In this issue of the journal, Keszler et al. (2014) focused on a highly innovative non-pharmacological strategy. Specifically, they were able to harness the power of near-infrared (NIR) light-emitting diodes (LEDs) to liberate nitric oxide (NO) in a manner that exerted a potent cardioprotective effect. The findings of Keszler et al. (2014) are exciting on several grounds. Not only did these authors expand our understanding of the mechanism by which NIR elicits cardioprotection, they convincingly documented its utility in the setting of diabetes mellitus. This achievement cannot be overstated given the failure of most other cardioprotective strategies,

including ischemic conditioning, in this setting.

"NO" LIBERATION BY LIGHT

NIR light has been used to protect neurons from methanol toxicity, stimulate angiogenesis, heal chemotherapy-induced mucositis, and reduce myocardial infarct size through NO-dependent signaling. The beneficial role of NO is documented by studies in which its inhibition was found to abrogate the cardioprotective effects of ischemic preconditioning. Moreover, several agents known to increase NO bioavailability (for example, phosphodiesterase inhibitors, glycerol trinitrate, and nicorandil) are all potent activators of cardioprotective signaling. Of note, NO triggers mK_{ATP} channel activation through a PKG-cGMP dependent pathway, whose protective effects are abolished by PKG inhibitors or NO scavengers (Costa et al., 2008). Moreover, NO reduces mitochondrial ROS levels and oxidative stress during IR injury by trapping superoxide and eliciting conformational changes that promote S-nitrosation of Complex I of the electron transport chain (Wink et al., 1993; Paolocci et al., 2001; Chouchani et al., 2013). As such, NO markedly attenuates ROS-mediated toxicity while elevated ROS levels act to suppress basal and agonist-induced NO release (Wink et al., 1993; Paolocci et al., 2001).

Nitric Oxide Synthases (NOS), the major enzymes that produce NO, are upregulated in response to preconditioning stimuli. Since NOS are functionally downregulated in the context of diabetes mellitus, the cardioprotective signaling pathways that are elicited by NO are severely compromised in this setting. To circumvent this important limitation, various groups have developed a clever strategy for liberating NO directly from heme-containing proteins using NIR light. The utility of this strategy in diabetes, however, remained largely unknown—at least until now.

In an elegant Research Topic (http://journal.frontiersin.org/ResearchTopic/1809) hosted by Aon and colleagues, Keszler et al. (2014) demonstrated that NIR-mediated cardioprotection, which is likely to be NO-dependent (Lohr et al., 2009), was surprisingly NOS-independent. Treatment of hearts with the NOS inhibitor L-NAME did not significantly alter the extent of protection as the reduction in infarct size remained virtually unchanged. Likewise, the infarct-sparing effects of NIR were neither abolished in endothelial NOS deficient mice nor in a well-established model of type-2 diabetes mellitus (*db/db* mice).

The exciting findings of Keszler et al. (2014) should spur investigators to examine the potential cardioprotective efficacy of NIR as a tool for remote preconditioning. If NIR is indeed effective even when applied to remote areas and/or organs, its clinical applicability and translatability would be markedly enhanced. Given the short half-life and high reactivity of NO, the maximum allowable distance between the site of NIR application and the infarct location should be carefully determined in future studies. Finally, since NO signaling modulates numerous cellular targets, including a host of sarcolemmal ion channels and calcium regulatory proteins, it will be critical to investigate the effects of NIR on electrophysiological properties and excitation-contraction coupling. As elegantly highlighted in this Research Topic, NIR is promising in its ability to treat diabetic hearts, for which classically cardioprotective therapies have failed. Indeed, the findings of Keszler et al. (2014) break new grounds in our effort to manage diabetic patients who are at high risk of ischemia-related complications.

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The calcium-ROS-pH triangle and mitochondrial permeability transition: challenges to mimic cardiac ischemia-reperfusion

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A commentary on

Differential effects of buffer pH on Ca2+−induced ROS emission with inhibited mitochondrial complex I and III

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Reperfusion of the heart following sustained ischemia is associated with enhanced reactive oxygen species (ROS) production, Ca^{2+} accumulation, and pH_i normalization that are the major inducers of mitochondrial permeability transition (mPT). Despite intensive studies, a cause-and-effect relationship between the ROS-Ca^{2+}-pH_i triangle and mPT has not yet been established (Halestrap et al., 2004; Bernardi, 2013). Initially, several proteins such as VDAC, ANT, and phosphate carrier (PiC) have been suggested as the essential structural components of the mPTP. However, genetic studies from different groups demonstrated that pore opening can occur in the absence of these proteins indicating that they are not involved in the mPTP structure (*reviewed in* Bernardi, 2013; Halestrap and Richardson, 2015). Emerging studies suggest that the mitochondrial F_0F_1-ATP synthase or electron transport chain (ETC) complex V is involved in pore formation and may actually play an important role as a structural component of the mPTP (Giorgio et al., 2013; Alavian et al., 2014; Azarashvili et al., 2014; Carraro et al., 2014). In addition to the unknown molecular identity of the mPT pore (mPTP), a lack of *in vitro* models mimicking cardiac ischemia-reperfusion (IR) makes it difficult to elucidate the precise role of mitochondrial ROS, Ca^{2+}, and pH_i in response to oxidative stress. Mitochondrial ETC complexes I, II, and III are the main sites of ROS (superoxide anion) production (**Figure 1**). Dysfunction of the complexes induced by cardiac IR enhances ROS which are not efficiently eliminated by the mitochondrial antioxidant system due to high ROS generation and low ROS scavenging. Activity of ETC complexes may be diminished by a number of factors including cardiolipin oxidation, degradation of supercomplexes, alteration of the ion homeostasis/redox potential, etc.

The article published by Lindsay et al. (2015) studies pH-dependence of Ca^{2+}-induced swelling (a marker of mPTP opening), ROS generation and respiratory function of isolated guinea pig cardiac mitochondria using substrates and inhibitors for ETC complexes I and III. Results of the study demonstrated that pH and Ca^{2+}-induced mPTP opening have different effects on ROS production at complexes I and III. The authors attempted to mimic cardiac IR by blocking complexes I and III with rotenone and antimycin in the presence of pyruvate and succinate, respectively. Although this is the only approach to assess the contribution of individual ETC complexes to ROS production in isolated mitochondria, it is rather different from the *in vivo* condition observed in cardiac IR. Each of complexes I and III contain two

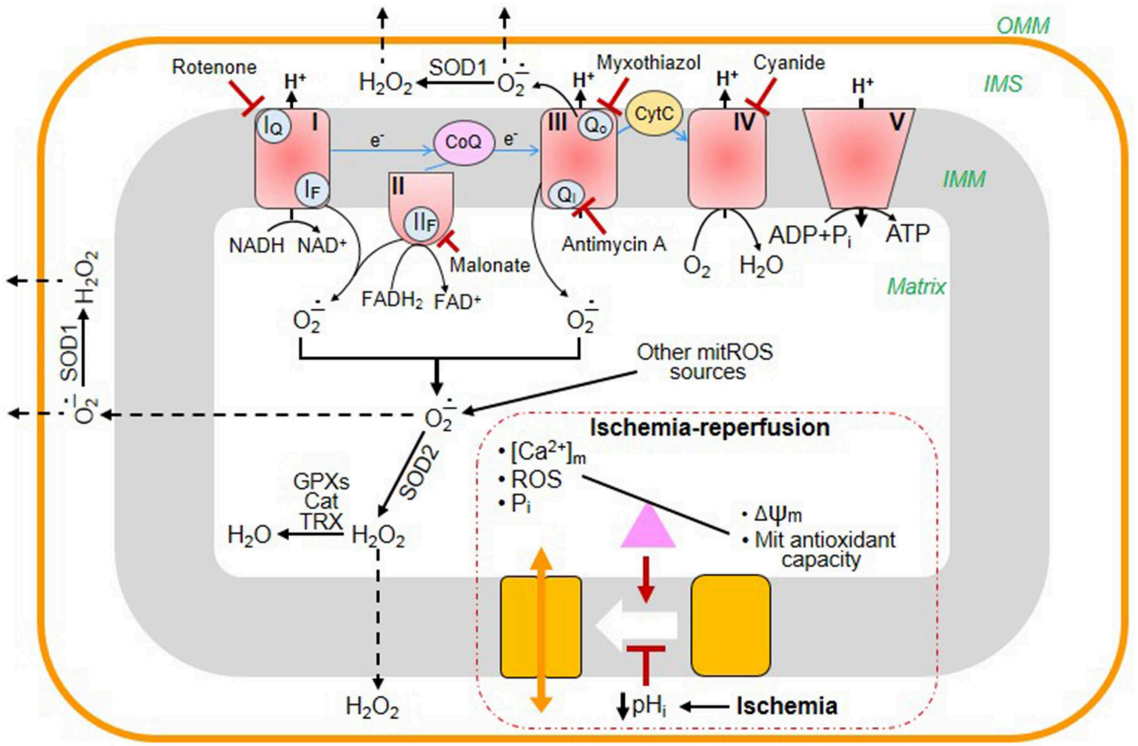

FIGURE 1 | Main sites of ROS generation in mitochondria and their elimination by the antioxidant system: the effects of cardiac ischemia-reperfusion (IR). The mitochondrial electron transport chain (ETC) consists of four multi-subunit complexes (I,II,III, and IV), coenzyme Q (CoQ), and cytochrome c (Cyt C). Electrons (e-) are transferred through the ETC from the reducing $NADH/FADH_2$ to O_2, finally generating H_2O at complex IV. The electron transfer through the ETC is accompanied by ROS generation at complexes I, II, and III. Complex I oxidizes NADH and produces superoxide ($O_2^{-\cdot}$) at the flavin site (I_F) and ubiquinone-binding site (I_Q) that is released to the matrix. Complex II oxidizes succinate to fumarate and generates $O_2^{-\cdot}/H_2O_2$ both in the reverse and forward reactions through the flavin site (II_F). Complex III is the main source of ROS that produces $O_2^{-\cdot}$ to both cytoplasm and matrix. Under normal physiological conditions, the level of mitochondrial ROS is regulated by mitochondrial and cytoplasmic antioxidant systems. Superoxide dismutases (SOD1, SOD2) convert $O_2^{-\cdot}$ into H_2O_2 which is then eliminated by catalase (Cat), glutathione peroxidases (GPXs), and reduced thioredoxin (TRX). During cardiac IR, the balance between generation and scavenging of ROS is altered due to high ROS production and low antioxidant capacity of mitochondria. Mitochondrial permeability transition pores (mPTP) remain closed during ischemia due to low pH_i. However, at reperfusion, excessive ROS generation together with increased $[Ca^{2+}]$ and $[P_i]_m$ and diminished membrane potential ($\Delta\psi_m$) cause mPTP opening. The blunted arrows indicate the sites where the inhibitors rotenone, malonate, myxothiazol, antimycin A, and cyanide bind to ETC complexes.

sites of ROS generation, and rotenone and antimycin inhibit only one site at complex I (the ubiquinone-binding site, I_Q) and complex III (the quinone-reducing center, Qi), respectively. Complete chemical blocking of these sites and the use of only one substrate (pyruvate or succinate) for each complex are the major limitations of the study. On the other hand, more recent studies revealed that succinate is a general metabolic marker of ischemia in a variety of tissues including the heart, and that it is responsible for mitochondrial ROS production during reperfusion by reverse electron transport at complex I Inhibition of ischemic succinate accumulation and its oxidation after subsequent reperfusion was sufficient to ameliorate *in vivo* cardiac IR injury in rodents (Chouchani et al., 2014). Indeed, in the study by Lindsay et al. (2015), the authors measured ROS levels at complex I-III, but not complex III alone. Most of the ROS signal observed during succinate oxidation is rotenone-sensitive and this is associated with the I_Q site of complex I due to the backflow of electrons from the reduced Q-pool. Accordingly, reverse electron transfer from the reduced QH_2 pool at site I_Q should be blocked to measure ROS generation solely at complex III.

Oxidative stress induces a complex of biochemical, biophysical and topographical changes of the inner mitochondrial membrane that ultimately result in malfunction of the ETC. The latter, when accompanied by membrane depolarization, ROS generation, matrix Ca^{2+} and P_i overload, can induce reversible (low conductance, physiological) or irreversible (high conductance, pathological) mPT depending on the severity of IR. Moreover, opening of the mPTP can further enhance the aforementioned alterations. Since low pH_i in the ischemic myocardium blocks the mPTP, pore opening occurs only upon reperfusion with normalization of pH_i (Griffiths and Halestrap, 1995). The contribution of each ETC complex may be different throughout the ischemic period due to changes in the redox potential, ion homeostasis, and antioxidant system of mitochondria. Mitochondrial respiration, ROS generation and mPTP opening were pH-dependent, which indicates that interactions between these parameters are

complex (Lindsay et al., 2015). ROS production at pH 6.5 was significantly lower than that at pH 6.9 and pH 7.15 for complexes I and I-III in mitochondria with Ca^{2+} swelling. Notably, significant mitochondrial swelling associated increased ROS generation was observed in the presence of succinate and antimycin A at all pH (6.5; 6.9, and 7.15), and both swelling and ROS production were significantly reduced by cyclosporin A to basic levels. These data confirm previous studies that mPTP opening induces mitochondrial ROS production (Batandier et al., 2004) and lowering pH inhibits pore opening (Javadov et al., 2008). However some findings of the study remain unanswered. For instance, Ca^{2+}-induced swelling was accompanied by a great increase in ROS release from complex I at pH 6.9 but not at pHs 7.15 or 6.5. Respiration rates (state three and RCI) were markedly affected at pHs 6.5 or 6.9 for complex I but not for complex I-III during Ca^{2+} swelling. Remarkably, inhibition of complex I by rotenone blocks PTP opening in tissues that express low levels of cyclophilin D, and, conversely rotenone does not affect the PTP in tissues which are characterized by high levels of expression of cyclophilin D and sensitivity to cyclosporin A (Li et al., 2012). The inhibitory effect of rotenone on PTP can complicate the interpretation of the results reported by Lindsay et al. (2015). In addition, it is very difficult to assess the effect of matrix pH on mPTP *in vitro* to mimic conditions observed *in situ*. First of all, the relevant parameter is matrix pH (Nicolli et al., 1993); in deenergized mitochondria the probability of pore opening has an optimum at matrix pH 7.4, although is changes both below and above this level. During IR, matrix pH is influenced by reenergization, and by the secondary events that follow transport of species that depend on the delta pH. This is critical for the PTP, because reenergization may offset the protective effects of an initially acidic matrix pH because of increased Pi uptake, and as shown in isolated brain mitochondria, ischemic and postischemic acidosis may worsen rather than relieve PTP-dependent tissue damage (Kristian et al., 2001).

In conclusion, despite certain limitations, the elucidation of the contribution of ROS and pH_i to mPTP opening via chemical inhibition of complexes I and III by Lindsay et al. (2015) opens new directions for further studies.

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