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Uncoupling protein 1 of brown adipocytes, the only uncoupler: a historical perspective

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Uncoupling protein 1 (UCP1), is a unique mitochondrial membranous protein devoted to adaptive thermogenesis, a specialized function performed by brown adipocytes. Whereas the family of mitochondrial metabolite carriers comprises ∼40 members, UCP1 is the only memberable to translocate protons through the inner membrane of brown adipocyte mitochondria. By this process, UCP1 uncouples respiration from ATP synthesis and therefore provokes energy dissipation in the form of heat while, also stimulating high levels of fatty acid oxidation. UCP1 homologs were identified but they are biochemically and physiologically different from UCP1. Thirty five years after its identification, UCP1 still appears as a fascinating component. The recent renewal of the interest in human brown adipose tissue makes UCP1 as a potential target for strategies of treatment of metabolic disorders.

Keywords: brown adipocyte, fatty acid, membranous carrier, mitochondria, proton transport, respiration coupling, thermogenesis, uncoupling

INTRODUCTION

FROM PHYSIOLOGICAL MEASUREMENTS OF THERMOGENESIS TO THE IDENTIFICATION OF UCP1

Basal thermogenesis results from the basal activity of many biochemical pathways including ATP-ases and futile cycles. Adaptive thermogenesis is regulated and occurs in particular conditions (cold exposure, arousing from hibernation, food intake. . .). This process involves the recruitment of different cell types and the activation of specific biochemical pathways. Any process that occurs without performing useful work, accumulating intermediates, or concentrating ions has an efficiency of zero from the standpoint of energy conservation or 100% for the purposes of thermogenesis (Nicholls and Locke, 1984). Although ATP-ases contribute, the largest part of heat production by cells probably comes from many metabolic pathways and in particular from oxidation of substrates.

In rodents or in newborns of some species, elegant *in vivo* studies in the 1960s established that brown fat depots were engaged in thermogenesis; this led to an increase in the temperature of the blood in brown fat which was rapidly distributed to heart, brain, kidney, and skeletal muscle (reviews in Nicholls and Locke, 1984; Cannon and Nedergaard, 1985, 2004; Himms-Hagen and Ricquier, 1998). The thermogenic activity of brown adipocytes was confirmed by microcalorimetric determinations of the heat output of excised tissue, isolated brown adipocytes, and isolated brown fat mitochondria (Nedergaard et al., 1977; Seydoux and Girardier, 1977; Ricquier et al., 1979). Physiological or pharmacological experiments established that the ability of animals (mainly rodents) to activate thermogenesis in response to cold exposure correlates with the amount of brown fat and to its activation by the sympathetic nervous system mediates and (Himms-Hagen, 1989).

Thermogenesis is dependent on oxygen consumption and therefore on the ability of cells to oxidize substrates in their mitochondria. Morphologically, brown adipocytes are very unique since they contain an extremely high number of mitochondria; these specialized mitochondria have a highly developed inner membrane, the membrane where the respiratory chain complexes are anchored. In other words, the morphology of brown adipocytes confers on these cells a very high capacity to oxidize substrates (**Figure 1**). Therefore, when heat is required (exposure to the cold, as an example), norepinephrine released by sympathetic nerves rapidly activates brown adipocytes resulting in fatty acid oxidation and heat production. Independently, Smith and Lindberg observed in 1967 that thermogenesis in brown adipose cells resulted from a weak coupling of respiration to ADP phosphorylation, leading to waste of oxidation energy as heat. Some years later, Nicholls and Ricquier showed the presence of a specific 32-kD protein in the inner mitochondrial membrane of brown adipocytes (**Figure 1**) that could uncouple respiration to produce heat rather than ATP (see reviews in Nicholls and Locke, 1984; Cannon and Nedergaard, 2004; Nedergaard et al., 2005). This protein was later termed uncoupling protein UCP and renamed uncoupling protein 1 (UCP1) when UCP2 was identified (Fleury et al., 1997). The obvious thermogenic activity of UCP1 in mitochondria was clearly demonstrated by Kozak et al. (1994) observing the cold sensitive phenotype of the *Ucp*1$^{-/-}$ mouse (Enerbäck et al., 1997). UCP1 activity and regulation are reviewed below.

UCP1: A SPECIFIC PROTON CARRIER UNCOUPLING RESPIRATION FROM ATP SYNTHESIS

UCP1 IS A RESPIRATION UNCOUPLER, SHUNTING THE NORMAL PROTON CIRCUIT AND IS PHYSIOLOGICALLY REGULATED

The physiological measurements of BAT thermogenic activity instigated the search for a mechanism unique to brown adipocyte mitochondria. The search for a respiration uncoupling mechanism, unique to these mitochondria came at a moment when Mitchell had proposed the – rather debated at that time – chemiosmotic theory; a theory explaining that the proton gradient and

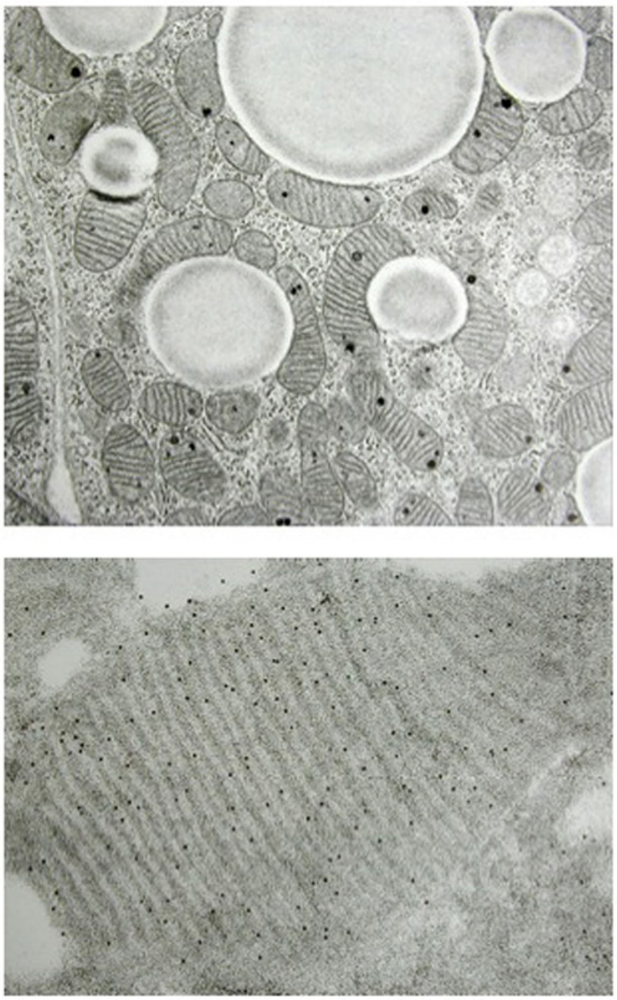

FIGURE 1 | Histology of brown adipocyte and presence of UCP1 in mitochondria. The cytosol of brown adipocytes is characterized by numerous mitochondria and lipid droplets (upper part). Magnification of a brown adipocyte mitochondrion showing parallel cristae and UCP1 detected using antibodies (black dots, lower part). (Figure kindly provided by Dr. Saverio Cinti, University of Ancona).

proton circuit through the inner membrane of mitochondria or chloroplasts, were governing ADP phosphorylation following respiration or exposure to light. According to Mitchell, oxidative phosphorylation is the process by which ADP phosphorylation by the mitochondrial ATP-synthase is coupled to mitochondrial oxygen consumption and to re-oxidation of reduced coenzymes via the proton electrochemical gradient generated by complexes I, III, and IV of respiratory chain. Oxygen then accepts electrons at the level of cytochrome-*c*-oxidase (complex IV) for conversion to water. Therefore, according to Mitchell, energy of the proton gradient ($\Delta\mu_{H+}$) , or the so-called proton-motive force Δp, is used to drive ATP synthesis by ATP-synthase. The proton gradient slows respiratory chain activity and facilitates ATP synthesis. The message here was that a proton circuit linking respiratory chain (protons out) and ATP-synthase (proton re-entry) operated such that oxygen consumption and ATP synthesis were tightly linked (**Figure 2**). Consequently, a proton leak in the inner mitochondrial membrane, distinct from the re-entry of protons via ATP-synthase, would lower the proton gradient and allow energy to be dissipated as heat. The reason for that is that down regulation of the proton gradient lowers the membrane potential which immediately activates the proton pumps and respiratory chain and provokes heat production since oxidation energy is not consumed by the ATP-synthase machinery. Actually, this is what happens when a chemical uncoupler is added to respiring liver or skeletal muscle mitochondria. Taken into consideration this postulate, Nicholls observed that isolated brown adipocyte mitochondria exhibit a very high and unique ion permeability in their inner membrane (review in Nicholls, 2006). This ion permeability, first detected as a chloride permeability, is a proton permeability strongly inhibited in presence of nucleotides, previously shown by Rafael and others to restore the respiratory control of brown adipocyte mitochondria (reviews in Cannon and Nedergaard, 2004; Nicholls, 2006). Photo-affinity labeling of hamster brown fat mitochondria with radioactive nucleotides was used by Heaton et al. (1978) to identify the uncoupling (proton transport) pathway as a 32-kD membranous protein. These data were in agreement with the previous description of this protein, when such a protein was (i) described to be present in rat brown fat mitochondria membrane but absent in liver mitochondria, (ii) the only membrane protein to be significantly increased in brown fat mitochondria following cold-adaptation, and (iii), down regulated when cold-exposed animals returned to the warm (Ricquier and Kader, 1976). Therefore, UCP1 not only plays an important physiological role in Nature, it also occupies a unique position among the family of membranous mitochondrial porters driven by oxidative metabolism: it is the exception that proves the rule of chemi-osmotic theory (Garlid and Jaburek, 1998; **Figure 2**).

UCP1 PROTON TRANSPORT ACTIVITY AND REGULATION

Nicholls developed a methodology to determine the current/voltage relationship of the basal proton leak of mitochondria by titrating down succinate respiration with malonate, allowing to calculate CmH^+, the proton conductance of the inner membrane of liver mitochondria in nanomole $H^+/min^{-1}/mg^{-1}, mv^{-1}$. Doing that with brown fat mitochondria, he found that the freshly prepared mitochondria had an enormous proton conductance and were incapable to maintain more than a few mV of proton-motive force whereas coupled mitochondria exhibit a membrane potential value close to 200 mV. It became clear that UCP1, when active, was acting as a proton translocator (Nicholls, 2006). When thermogenesis is not required, nucleotides bind UCP1 and inhibit its activity. In presence of inhibitory nucleotides, UCP1 has no residual proton conductance and is not leaky (Shabalina et al., 2010). From physiology physiology perspective, the search for a natural activator of UCP1 was logical. When thermogenesis is physiologically required, norepinephrine released by surrounding sympathetic fibers activates lipolysis which increases the level of free fatty acids in brown adipocyte mitochondria. The free fatty acids not only act as substrates for oxidation but also activate UCP1. The demonstration of the major ability of free fatty acids to activate UCP1 came from experiments where albumin

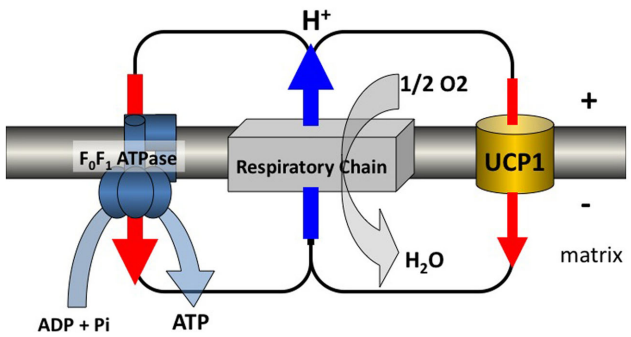

FIGURE 2 | Proton circuit. UCP1 is inserted in the mitochondrial inner membrane where, also present, is a multienzymatic complex called the respiratory chain made of complexes I to IV. The respiratory chain reoxidizes reduced coenzymes and electrons are driven to oxygen. This oxido-reduction step liberates energy which is used to generate an electrochemical gradient of protons across the inner membrane. This gradient is normally consumed by the ATP-synthase which phosphorylates ADP. UCP1 transports protons passively and makes possible a futile cycle of protons across the inner membrane leading to increased energy expenditure. This schema illustrates the situation encountered in brown adipocytes of mammals where a large amount of respiratory chains as well as a large amount of UCP1 are present. Activation of the futile cycling increases considerably energy expenditure and thus heat production by these thermogenic cells. In other cells where homologs of the UCP1 are expressed at much lower level, this pathway would represent a minor contributor to energy expenditure, but might be of importance to avoid oxidative damage (Figure kindly designed by Frédéric Bouillaud).

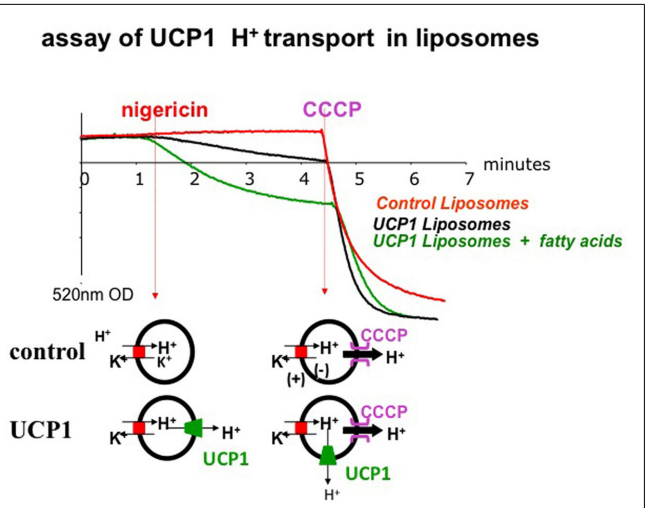

FIGURE 3 | Reconstitution of the proton transport activity of purified UCP1 in liposomes (Mozo et al., 2006). Membrane potential ($\Delta\Psi$) is appreciated using a safranin probe sensitive to membrane potential and allowing a measurement of optical density at 520 nm. In presence of nigericin, H^+ is exchanged against K^+ (H^+ in, K^+ out), there is no charge exchange but a ΔpH is generated. In liposomes containing UCP1, UCP1 dissipates this ΔpH gradient and also increases $\Delta\Psi$ inducing the polarization of the membrane (and a decrease of O.D. signal). The slope of the decrease of the safranin signal is an assay of UCP1 protonophoric activity. Addition of fatty acids to liposome containing UCP1 markedly polarizes the membrane due to activation of the protonophoric activity of UCP1. Upon addition of CCCP, a strong protonophor, H^+ ions go out and positively polarize the liposome membrane on external side.

for free fatty acids were added to isolated brown adipocytes cells and mitochondria. For example, in an elegant experiment, brown adipocyte mitochondria were slowly infused with palmitate to mimic lipolysis. During this procedure, the CmH^+ of UCP1 increased dramatically, the membrane potential decreased, and respiration rose sharply. (Nicholls and Locke, 1984; Ledesma et al., 2002; Cannon and Nedergaard, 2004; Nicholls, 2006). Comparing *wt* and *Ucp*1$^{-/-}$ mice brown fat mitochondria and using a non-metabolizable fatty acid analog, the Stockholm group confirmed the importance of UCP1 in thermogenesis and demonstrated that neither the ability to be metabolized, nor an innate uncoupling activity was a necessary property of UCP1 activators (Shabalina et al., 2008).

Other demonstrations of the proton transport activity of UCP1 were made by researchers who reconstituted its activity in liposomes (see an example in **Figure 3**). In such conditions, free fatty acids activate the proton translocating activity of UCP1 whereas nucleotides (GDP, GTP, ADP, ATP) inhibit it (Strieleman et al., 1985; Winkler and Klingenberg, 1994). According to these data, UCP1 is a purely H^+ translocating protein, the protons being translocated in a carrier-like fashion, instead of by a H^+ channel through the membrane. In other respects, there is still a debate whether fatty acids, (i) only activate proton transport (González-Barroso et al., 1998; Ledesma et al., 2002; Mozo et al., 2006), (ii) participate as a prosthetic group that delivers protons to a site where they are translocated to the matrix, or (iii) are transported as anions by UCP1 as a part of a cycling mechanism completed with the translocation of the protonated form across the lipid bilayer, with a net uptake of a proton (Skulachev, 1988; Garlid and Jaburek, 1998).

UCP1 AA ACID SEQUENCE AND PREDICTED STRUCTURE

The amino-acid sequence of UCP1 was determined following purification by Klingenberg (2010) and Aquila et al. (1985). It was also predicted from the sequencing of cloned cDNA (Bouillaud et al., 1985, 1986). Clearly, UCP1 is partly homologous to members of the anion mitochondrial carriers protein family (also referred to as the metabolite transporters of the mitochondrial inner membrane), including the ADP/ATP carrier and the phosphate carrier. Moreover, like the ADT/ATP carrier and mitochondrial carriers, UCP1 has a tripartite structure comprising three similar sequences of ~100 residues each. Hydropathy plot analysis predicted the existence of six membrane-spanning α-helices (Aquila et al., 1985; Bouillaud et al., 1986). This prediction was supported by an immunological analysis of antigenic sites in UCP1 (Miroux et al., 1993). UCP1 structure has not been resolved yet but is probably close to the structure of the adenine nucleotide translocator (Pebay-Peyroula et al., 2003) and the structure of UCP2 recently identified (Berardi et al., 2011).

UCP1 IS SPECIFIC FOR BROWN ADIPOCYTES AND IS VERY ABUNDANT

The specific activity of UCP1 is unique to brown adipocytes. This was fully demonstrated in mice made null for *Ucp*1, which became hypothermic in a cold ambiance (Enerbäck et al., 1997; Nedergaard et al., 2001). It was also confirmed by recombinant

expression of UCP1 in yeasts or mammalian cells (Casteilla et al., 1990; Bouillaud et al., 1994; González-Barroso et al., 1998). Many experimental approaches including UCP1 mRNA detection or UCP1 immunodetection, as well as the analysis of the activity of a reporter gene drived by *Ucp*1 promotor in transgenic mice, confirmed that *Ucp*1 expression is only observable in brown adipocyte (Cassard-Doulcier et al., 1998; Ricquier and Kozak, 2003).

An additional feature of UCP1 is that it is markedly abundant in brown adipocyte mitochondria where it comprises up to 8% of the total protein. The reason for such an amount is unknown but suggests that UCP1 molecular activity is rather weak.

UCP1 GENE: SPECIFIC EXPRESSION IN BROWN ADIPOCYTES AND REGULATION OF TRANSCRIPTION

CLONING OF CDNAS AND GENE, GENE ORGANIZATION IN RODENTS AND IN HUMANS, TRANSCRIPTIONAL REGULATION

The question of transcriptional regulation of *Ucp*1 has two sides: its unique expression in brown adipocytes, and, the transcriptional activation by norepinephrine and other non-cell-autonomous factors like T_3. In fact, it is not easy to list *cis* elements and *trans*-regulatory factors regulating, either the level of transcription of the gene, or the cell-specific transcription since these two mechanisms are probably controlled by the same factors. UCP1 biosynthesis is largely controlled at the level of transcription which is sharply activated within minutes after exposure of rodents to the cold (Ricquier et al., 1984, 1986; Ricquier and Kozak, 2003). The sympathetic activation of brown adipocytes and the subsequent and immediate rise in cAMP is the primary and main trigger of *Ucp*1 transcription, but other factors such as T_3 (Silva and Rabelo, 1998) and retinoic acid (Alvarez et al., 1995; Larose et al., 1996; Rabelo et al., 1996; Gonzalez-Barroso et al., 2000a) are critical for a full physiological response (Gonzalez-Barroso et al., 2000b). The molecular mechanisms involved in the regulation of rodent and human *UCP*1 transcription have been partially elucidated and a critical 200-bp *cis* region (and more precisely the moiety of this region) having an enhancer activity, located a few kb upstream of the transcriptional start was identified (Cassard-Doulcier et al., 1993; Kozak et al., 1994; Gonzalez-Barroso et al., 2000a). This region is able to bind transcriptional factors such as CREB, CCAAT/enhancer binding proteins α and β, jun, Ets1, thyroid hormone receptors, retinoid X-receptor, and PPARs. Other important regions, in particular cAMP-response elements, were also identified outside of the enhancer region and in the promoter region (Cassard-Doulcier et al., 1994; Kozak et al., 1994; Yubero et al., 1994, 1998; Alvarez et al., 1995; Silva and Rabelo, 1998; Rim and Kozak, 2002; Xue et al., 2005). Xue et al. (2005) proposed that small variations in the levels of several transcriptional components of the *Ucp*1 enhanceosome interact synergistically to achieve large differences in *Ucp*1 expression. In addition to these transcription factors, the co-activator PGC-1α also plays an important role (Puigserver et al., 1998).

GENETIC STUDIES IN HUMAN COHORTS

The human UCP1 gene was mapped to the long arm of chromosome 4 in q31 region (Cassard et al., 1990). A *Bcl*1 polymorphic site was identified at bp-3826 upstream of the TATA box of the *UCP*1 promoter in Bouchard's laboratory (Oppert et al., 1994). Several studies of association of this polymorphism were conducted and revealed that the *UCP*1 A-3826G polymorphism is not a major contributor to obesity development, however, the main observation was a significant association of this polymorphism with fat gain over time (Oppert et al., 1994; Gonzalez-Barroso et al., 2000b).

THE NOVEL UCPS, UCP2, AND UCP3 ARE BIOCHEMICALLY AND PHYSIOLOGICALLY DISTINCT FROM UCP1

UCP2 and UCP3, two Homologs of UCP1, were described in 1997 (Boss et al., 1997; Fleury et al., 1997; Vidal-Puig et al., 1997). Initially, the high level of amino-acid similarity with UCP1 (these new proteins display 57% identity with UCP1) as well as functional assays in yeast were in favor of an uncoupling activity (Fleury et al., 1997; Rial et al., 1999). However, a large number of studies based on physiological measurements of UCP2 or UCP3 expression (such as a marked up-regulation of these two UCPs in skeletal muscles of rodents and humans upon starvation Bevilacqua et al., 2005; Millet et al., 2007) and on analysis of mice null for *Ucp*1 or *Ucp*2, contradicted the first view (Boss et al., 2000; Ricquier and Bouillaud, 2000; Stuart et al., 2001; Rousset et al., 2004). Presently, it is difficult to consider UCP2 or UCP3 as membranous carriers able to uncouple respiration similarly to UCP1 (Nedergaard et al., 1999; Nedergaard and Cannon, 2003). UCP1 is present in a unique tissue the function of which is thermogenesis, whereas UCP2 is widely expressed in tissues and cells (gut, lung, brain, pancreatic islets, immune cells...) and UCP3 is present in skeletal muscles and brown adipose tissue. It appears that these other UCPs are metabolite transporters of the inner mitochondrial inner membrane and can limit the level of reactive oxygen species (Arsenijevic et al., 2000; Harper and Gerrits, 2004). UCP2 is able to inhibit glucose-induced insulin release (Zhang et al., 2001) and it has a transport activity directly or undirectly favoring glucose sparing and fatty acid oxidation (Pecqueur et al., 2008, 2009; Bouillaud, 2009).

CONCLUSION AND PROSPECTIVES: SEARCH FOR ACTIVATORS OR INDUCERS OF UCP1, IDENTIFICATION OF COMPONENTS MIMICKING UCP1, INDUCTION OF THERMOGENIC BROWN ADIPOCYTES

The brown adipocytes remain the cells uniquely able to rapidly burn fatty acids and dissipate oxidation energy as heat. Their activity is strictly dependant on their high content of mitochondria and above all on the presence of UCP1, a very particular membranous carrier, able to disrupt the respiratory-induced $\Delta\mu_H^+$ via a physiologically regulated proton transport activity. Therefore, these cells and UCP1 in particular, offer a chance to find compounds that increase fatty acid oxidation in obese patients and also in patients with metabolic syndrome.

Consequently, understanding the mechanisms which regulate transcription and expression of the human *UCP*1 will facilitate the identification of molecules able to increase the levels of this protein in order to elevate energy expenditure in adult patients. Another approach will be to activate the UCP1 protein itself by searching for specific activators solely interacting with UCP1 (Rial et al., 2011). This is certainly a difficult aim, but since free fatty acids

activate UCP1, modified fatty acids could be engineered. However, such new molecules should be totally specific for UCP1. Another strategy will be to identify chemical compounds able to induce a very slight uncoupling of respiration in tissues such as muscles. However, this later approach is very risky since induction of respiration uncoupling, even moderate, in cells other than brown adipocytes, may lower ATP synthesis and therefore be extremely deleterious. Remember that the use of classical chemical uncoupler, such as 2,4-dinitrophenol, caused serious illness in imprudent individuals and must be strictly banned (Grundlingh et al., 2011). Finally, an interesting approach would be to facilitate the emergence of new brown adipocytes from precursors present in skeletal muscles or in white adipose depots (Seale et al., 2007; Crisan et al., 2008).

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