



Docosahexaenoic acid reduces the incidence of early afterdepolarizations caused by oxidative stress in rabbit ventricular myocytes

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Accumulating evidence has suggested that ω 3-polyunsaturated fatty acids (ω 3-PUFAs) may have beneficial effects in the prevention/treatment of cardiovascular diseases, while controversies still remain regarding their anti-arrhythmic potential. It is not clear yet whether ω 3-PUFAs can suppress early afterdepolarizations (EADs) induced by oxidative stress. In the present study, we recorded action potentials using the patch-clamp technique in ventricular myocytes isolated from rabbit hearts. The treatment of myocytes with H_2O_2 (200 μ M) prolonged AP durations and induced EADs, which were significantly suppressed by docosahexaenoic acid (DHA, 10 or 25 μ M; $n=8$). To reveal the ionic mechanisms, we examined the effects of DHA on L-type calcium currents (I_{CaL}), late sodium (I_{Na}), and transient outward potassium currents (I_{to}) in ventricular myocytes pretreated with H_2O_2 . H_2O_2 (200 μ M) increased I_{CaL} by 46.4% from control (-8.4 ± 1.4 pA/pF) to a peak level (-12.3 ± 1.8 pA/pF, $n=6$, $p < 0.01$) after 6 min of H_2O_2 perfusion. H_2O_2 -enhanced I_{CaL} was significantly reduced by DHA (25 μ M; -7.1 ± 0.9 pA/pF, $n=6$, $p < 0.01$). Similarly, H_2O_2 -increased the late I_{Na} (-3.2 ± 0.3 pC) from control level (-0.7 ± 0.1 pC). DHA (25 μ M) completely reversed the H_2O_2 -induced increase in late I_{Na} (to -0.8 ± 0.2 pC, $n=5$). H_2O_2 also increased the peak amplitude of and the steady state I_{to} from 8.9 ± 1.0 and 2.16 ± 0.25 pA/pF to 12.8 ± 1.21 and 3.13 ± 0.47 pA/pF respectively ($n=6$, $p < 0.01$, however, treatment with DHA (25 μ M) did not produce significant effects on current amplitudes and dynamics of I_{to} altered by H_2O_2 . In addition, DHA (25 μ M) did not affect the increase of intracellular reactive oxygen species (ROS) levels induced by H_2O_2 in rabbit ventricular myocytes. These findings demonstrate that DHA suppresses exogenous H_2O_2 -induced EADs mainly by modulating membrane ion channel functions, while its direct effect on ROS may play a less prominent role.

Keywords: docosahexaenoic acid, H_2O_2 , early afterdepolarizations, reactive oxygen species, L-type calcium channel, sodium channel

INTRODUCTION

Extensive studies on the potential effects of fish oil omega-3 poly unsaturated fatty acids (ω -3 PUFA) on cardiac rhythm have provided controversial results (von Schacky, 2008). While some interventional studies reported either no effect or even promotion of arrhythmias in some subgroups of patients with heart disease (Raitt et al., 2005; Coronel et al., 2007; Den Ruijter et al., 2007; Cheng and Santoni, 2008), other studies have reported beneficial effects of ω -3-PUFAs on cardiac rhythm resulting in a reduction in the incidence of sudden cardiac death or mortality (London et al., 2007; Cheng and Santoni, 2008; Nodari et al., 2009). It seems that fish oil fatty acids may exert either pro- or anti-arrhythmic effects, probably depending on different underlying mechanisms for the arrhythmias. Recent studies have also shown ω -3-PUFAs suppress afterdepolarizations and triggered activities induced by K channel blockers or by β -adrenergic stimulation in failing hearts

(Den Ruijter et al., 2006, 2008; Berecki et al., 2007; Smith et al., 2009). However, it is unclear whether ω -3-PUFAs have protective effects on arrhythmias induced by oxidative stress. Reactive oxygen species (ROS) have recently been implicated in the pathogenesis of cardiac arrhythmia during ischemic-reperfusion, aging, and heart failure. Oxidative stress caused by exogenous H_2O_2 induces early afterdepolarizations (EADs) and delayed afterdepolarizations (DADs) that may in turn trigger lethal arrhythmias. These afterdepolarizations are a result of a net increase in inward current, which is induced by activation of late sodium current (I_{Na}) and the L-type calcium current (I_{CaL}) via oxidized Ca^{2+} /Calmodulin-Dependent Protein Kinase II (CaMKII; Ward and Giles, 1997; Xie et al., 2009; Zhao et al., 2011). Our most recent study suggested that the transient outward potassium current (I_{to}) may also facilitate EAD generation by H_2O_2 (Zhao et al., 2012b). In the present study, we aim to assess the effects of docosahexaenoic acid (DHA, one of

ω -3-PUFAs) on exogenous H_2O_2 -induced EADs, and to further reveal potential underlying ionic mechanisms.

MATERIALS AND METHODS

This investigation conforms to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85–23, Revised 1996). All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey-New Jersey Medical School and by the Ethical Committee of Xi'an Jiaotong University. All experiments were performed at 35–37°C.

CELL ISOLATION

Ventricular myocytes were enzymatically isolated from the hearts of New Zealand white rabbits (male, 1.8–2.5 kg) as described previously (Xie et al., 2009). Briefly, after rabbits were anesthetized with intravenous pentobarbital hearts were removed and perfused retrogradely at 37°C in Langendorff fashion with nominally Ca^{2+} -free Tyrode's solution containing 1.4 mg/mL collagenase (Type II; Worthington) and 0.1 mg/ml protease (type XIV, Sigma) for 25–30 min. The hearts were removed from the perfusion apparatus after washing out the enzyme solution, the left ventricles were gently teased apart with forceps in a Petri dish and the myocytes were filtered through a nylon mesh. The Ca^{2+} concentration was gradually increased to 1.8 mM, and the cells were stored at room temperature and used within 8 h.

ELECTROPHYSIOLOGICAL RECORDING

Myocytes were current- or voltage-clamped using the perforated whole-cell patch-clamp technique (240 μ g/ml amphotericin B; Rae et al., 1991) for recordings of action potential, or $I_{Ca,L}$, I_{to} , and late I_{Na} . Voltage or current signals were measured with a MultiClamp 700A patch-clamp amplifier controlled by a personal computer using a Digidata 1322 acquisition board driven by pCLAMP 10 software (Molecular Devices, Sunnyvale, CA, USA).

To record action potentials (APs), patch pipettes (resistance 2–4 M Ω) were filled with an internal solution containing (in mM): 110 K-aspartate, 30 KCl, 5 NaCl, 10 HEPES, 0.1 EGTA, 5 MgATP, 5 Na_2 -phosphocreatine, 0.05 cAMP, pH was adjusted to 7.2 with KOH. The cells were superfused with Tyrode's solution containing (in mM): 136 NaCl, 4.0 KCl, 0.33 Na_2PO_4 , 1.8 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES, pH was adjusted to 7.4 with NaOH. APs were elicited with 2 ms, 2 to 4 nA square pulses at a pacing cycle length (PCL) of 6 s.

To record the $I_{Ca,L}$, patch pipettes (2–4 M Ω) were filled with an internal solution containing (in mM): 110 Cs-Aspartate, 30 CsCl, 5 NaCl, 10 HEPES, 0.1 EGTA, 5 MgATP, 5 Na_2 -phosphocreatine, 0.05 cAMP, pH 7.2 with CsOH, and the cells were perfused with a modified Tyrode's solution, in which KCl was replaced with CsCl. The myocytes were stimulated at a PCL of 6 s with a double-pulse protocol. Following a 100-ms prepulse to –40 mV from the holding potential of –80 mV (to inactivate Na^+ current and T-type Ca^{2+} current), $I_{Ca,L}$ was elicited by a subsequent test depolarization step to 0 mV for 300 ms.

Late I_{Na} was measured as described previously (Song et al., 2006). Glass pipettes (1–2 M Ω) were filled with an internal solution containing (in mM): 110 Cs-Aspartate, 30 CsCl, 10 HEPES,

0.5 EGTA, 0.2 Na_3 -GTP, 5 Na_2 -phosphocreatine-, 5 MgATP, pH 7.2 was adjusted with CsOH. Myocytes were bathed with a modified Tyrode's solution in which KCl was replaced with CsCl. Nifedipine (30 μ M) was added to the bath solution to block calcium channels. Late I_{Na} was elicited by 300 ms voltage-clamp pulses from –90 to –30 mV at a PCL of 6 s from a holding potential of –80 mV.

To record I_{to} , the pipette and superfusion solutions were the same as those for AP recording. Tetrodotoxin (TTX, 10 μ M) and $CdCl_2$ (0.5 mM) were added into the Tyrode's solution to inhibit I_{Na} and $I_{Ca,L}$. I_{to} was evoked by 400 ms depolarizing pulses to test potentials between –40 and +50 mV (0.1 Hz). The holding potential was set at –80 mV and a 100 ms prepulse was applied to –60 mV to inactivate the I_{Na} . I_{to} recovery from inactivation was investigated using a conventional two-pulse protocol: an inactivating pulse depolarizing to +50 mV for 400 ms (P1) followed by a variable recovery interval and subsequent +50 mV test pulse (P2). The inactivation of I_{to} and recovery from inactivation were best fit with a double exponential equation. All electrophysiological data were normalized as current densities by dividing measured current amplitude by whole-cell capacitance.

All chemicals were purchased from Sigma-Aldrich unless indicated. Because DHA is very sensitive to oxidation, DHA (Sigma-Aldrich) was dissolved in 100% ethanol under N_2 and kept at –20°C in the dark. Immediately before use, the DHA stock solution was diluted in the bath solution to reach the final concentrations needed. The maximum final concentration (0.1%) of ethanol had no effect on membrane currents.

MEASUREMENT OF INTRACELLULAR ROS

The myocytes were incubated with 5 μ M C-DCDHF-DA-AM (Invitrogen) for 30 min. C-DCDHF-DA is oxidized by ROS to dichlorofluorescein (DCF). ROS fluorescence (emission: ~530 nm) was measured by a 200 ms-exposure (excitation: ~480 nm) every 30 s using the Andor Ixon charge-coupled device camera. Recordings were started after a stable baseline was achieved.

STATISTICAL ANALYSIS

Data are presented as mean \pm SEM. Differences were tested for statistical significance by using paired or unpaired Student's *t* tests, with *p* < 0.05 considered significant.

RESULTS

DHA SUPPRESSES THE EADs INDUCED BY H_2O_2

Action potentials were recorded from single ventricular myocytes isolated from rabbit hearts using the perforated whole-cell patch-clamp technique under current-clamp mode. In order to reliably induce EADs, the cells were paced at a PCL of 6 s based on our previous studies (Sato et al., 2009; Xie et al., 2009; Zhao et al., 2012a). The average APD₉₀ of rabbit ventricular myocytes is 266 \pm 23 ms (*n* = 8) at base line. After APD and morphology reached steady state, the cells were perfused with 200 μ M H_2O_2 until EADs consistently appeared. Consecutively, DHA at either 10 or 25 μ M was added in the presence of H_2O_2 . The sudden and dramatic increase in APD₉₀ in **Figure 1A** indicates the incidence of EADs. As shown in **Figures 1A,B**, EADs were consistently induced by H_2O_2 at 5 min after perfusion. DHA (25 μ M) shortened the APD

prolongation from 894 ± 78 ms to 278 ± 52 ms, and significantly suppressed the frequency of EADs induced by H_2O_2 . The incidence of EADs was assessed by counting the number of EADs within 10 APs (from eight cells) in control, after H_2O_2 (200 μ M) and H_2O_2 (200 μ M) + DHA (at 10 or 25 μ M). The incidence of EAD was suppressed in all tested cells ($n = 8$), five of which showed complete abolishment of EADs after 3–5 min of treatment with 25 μ M DHA. As summarized in **Figure 1C**, the incidence of H_2O_2 -induced EADs were significantly reduced by direct perfusion of DHA at both 10 and 25 μ M, in a dose-dependent manner ($p < 0.05$ and $p < 0.01$, respectively, Fisher's exact test).

INHIBITORY EFFECT OF DHA ON I_{CaL} ENHANCED BY H_2O_2

Our previous studies have shown that reactivation of I_{CaL} plays a key role in H_2O_2 -induced EAD in rabbit ventricular myocytes (Xie et al., 2009; Song et al., 2010). Therefore, we first assessed the potential involvement of I_{CaL} in the inhibitory effect of DHA on H_2O_2 -induced EADs. I_{CaL} was recorded in rabbit ventricular myocytes using the perforated whole-cell patch-clamp technique under voltage-clamp mode. As shown in **Figure 2A**, H_2O_2 (200 μ M) gradually increased the amplitude of I_{CaL} at both peak and late phases (at ~ 250 ms), which reached the steady state at 5–7 min, consistent with the time course for EAD induction as shown in **Figure 1**. The I-V relations for the peak current (**Figure 2B**) showed that the I_{CaL} amplitude was pronouncedly increased at testing potentials -10 to $+40$ mV. For example a 46.4% enhancement was caused at 0 mV, i.e., from -8.4 ± 1.4 to -12.3 ± 1.8 pA/pF ($n = 6$, $p < 0.01$). DHA (25 μ M) significantly suppressed/reversed the elevation of the I_{CaL} amplitude (e.g., to -7.1 ± 0.9 pA/pF at 0 mV; $n = 6$, $p < 0.01$ compared to H_2O_2 -induced effect). In order to test the DHA effect on I_{CaL} under normal membrane potential conditions, we also performed AP-clamp experiments. As shown in **Figures 2C,D**, DHA markedly decreased both the peak and the late phase of I_{CaL} , which were enhanced by H_2O_2 , under AP-clamp conditions.

INHIBITORY EFFECT OF DHA ON LATE SODIUM CURRENT INCREASED BY H_2O_2

Since the activation of late I_{Na} also contributes to EAD generation induced by H_2O_2 (Ward and Giles, 1997; Xie et al., 2009), we next evaluated the effect of DHA on H_2O_2 -enhanced late I_{Na} . Late I_{Na} was elicited by 300 ms voltage-clamp pulses from -90 to -30 mV at a PCL of 6 s. The magnitude of late I_{Na} was evaluated by integration of the area ($nA \times ms = pC$) of the current over the last 50 ms of the -30 mV depolarizing pulse, using the integration (area) feature of the pCLAMP program. As shown in **Figure 3**, the late current component was significantly enhanced by H_2O_2 (200 μ M) from -0.7 ± 0.1 pC to -3.2 ± 0.3 pC ($n = 5$, $p < 0.01$) at 4–6 min after perfusion, when it reaches steady state level. This elevation was completely suppressed by Tetrodotoxin (TTX, 10 μ M), a selective I_{Na} inhibitor, confirming this late sustained inward current is due to late I_{Na} , although we cannot exclude minor contaminations on the baseline current from other currents such as Na-Ca exchange current (I_{NCX}), I_{CaL} or leaky

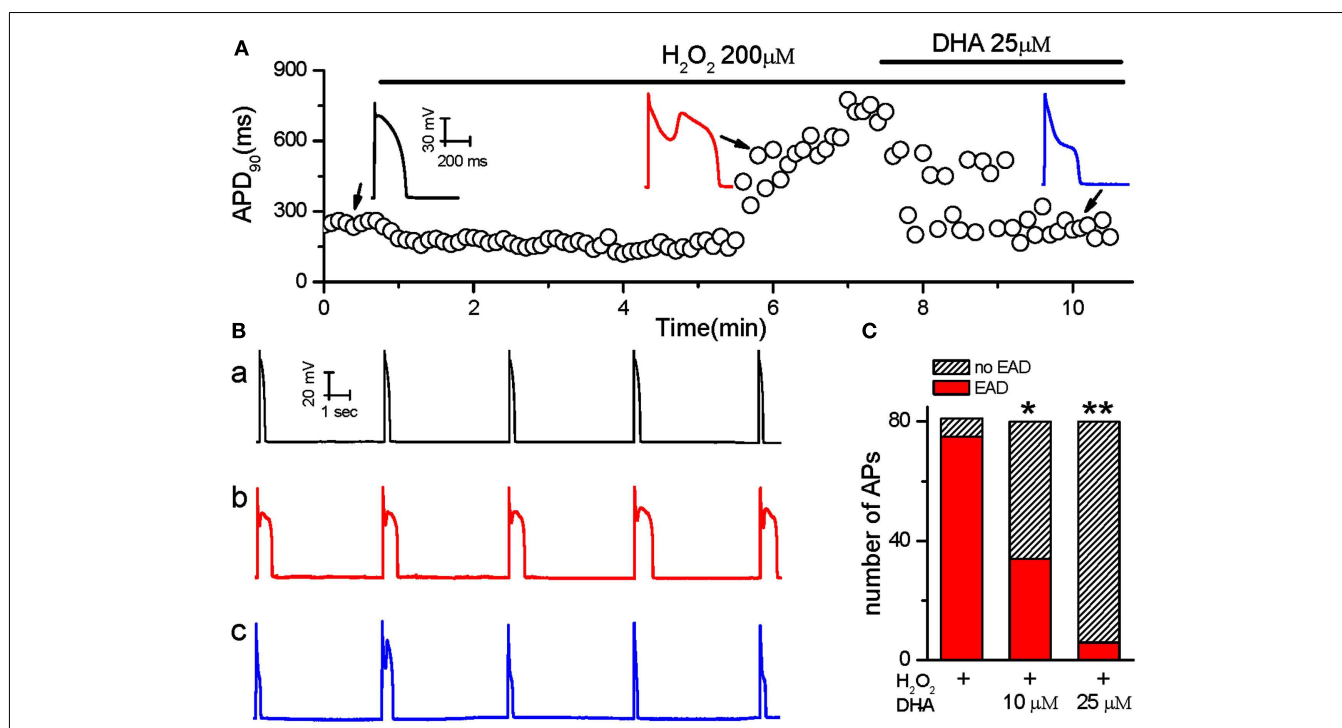


FIGURE 1 | The inhibitory effects of DHA on Early afterdepolarizations (EADs) induced by H_2O_2 . (A) Values of consecutive APD_{90} are plotted over time. The ventricular myocyte was treated with H_2O_2 and DHA as indicated by the horizontal bars above the plot. Three representative AP recordings under different conditions are shown in the insets. (B) Five

consecutive AP recordings from a cell exposed to control perfusate (a), 200 μ M H_2O_2 (b) and 200 μ M H_2O_2 + 25 μ M DHA (c). (C) Summarized bar graph showing dose-dependent inhibitory effects of DHA on the incidence of EADs induced by H_2O_2 ($n = 8$ cells). * $p < 0.05$, ** $p < 0.01$; Fisher's Exact Test vs. H_2O_2 .

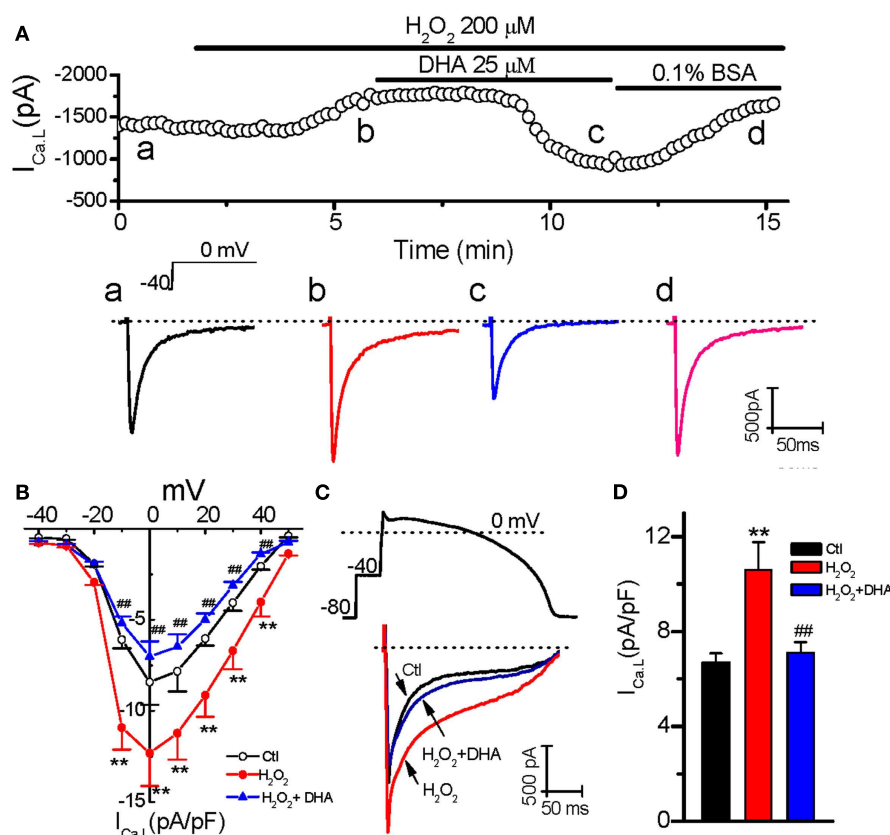


FIGURE 2 | Inhibitory effects of DHA on I_{CaL} enhanced by H_2O_2 . (A) Time course of peak I_{CaL} in a myocyte treated with $200 \mu M H_2O_2$ in the absence and presence of $25 \mu M$ DHA, and 0.1% bovine serum albumin (BSA). Representative traces of I_{CaL} corresponding to points a–d are shown under the plot. (B) The current-voltage relations for peak I_{CaL} from six cells treated with $200 \mu M H_2O_2$ in the absence and presence of $25 \mu M$ DHA. Test potentials ranged from -40 to $+50$ mV in 10 mV

steps. (C) An AP-clamp waveform (above) and superimposed current traces showing I_{CaL} under control (Ctl), in the presence of $200 \mu M H_2O_2$, and DHA($25 \mu M$) + H_2O_2 are shown respectively. (D) The late phase currents measured at 30 – 150 ms after the upstroke in (C) were summarized showing an inhibitory effect of DHA on the enhancement of I_{CaL} by H_2O_2 ($n=6$). ** $p < 0.01$ vs. control; ## $p < 0.01$ vs. H_2O_2 group.

current. H_2O_2 -increased late I_{Na} was effectively attenuated by $25 \mu M$ DHA (to -0.8 ± 0.2 pC at 2 – 4 min after DHA application, $n=5$, $p < 0.01$).

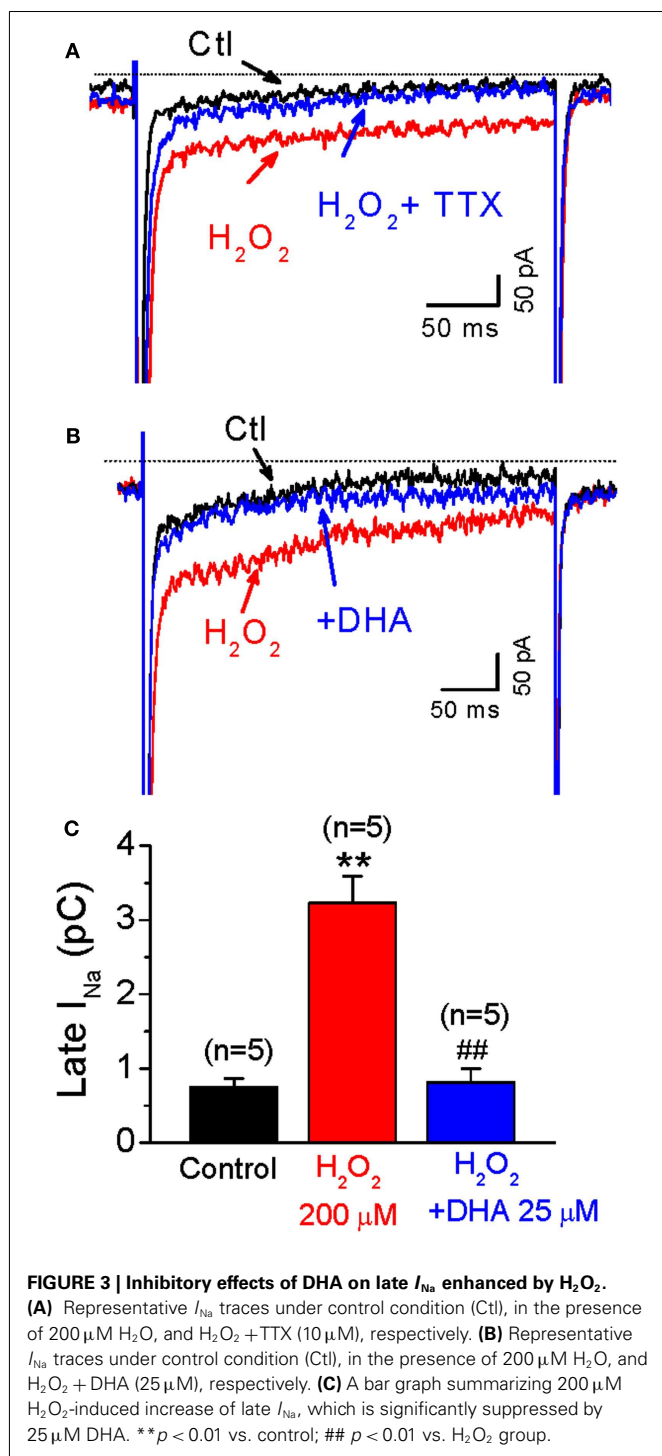
EFFECT OF DHA ON I_{T0} INCREASED BY H_2O_2

Consistent with our recent finding (Zhao et al., 2012b), H_2O_2 ($200 \mu M$) increased the amplitudes of both peak (from 8.94 ± 1.07 to 12.8 ± 1.21 pA/pF at testing potential of 50 mV, $n=6$, $p < 0.01$) and steady state (late phase at the end of 400 ms pulse; from 2.16 ± 0.25 to 3.13 ± 0.47 pA/pF, $n=6$, $p < 0.01$) component of I_{T0} . Additionally, H_2O_2 also slowed inactivation ($\tau_{s, in}$ from 96.6 ± 4.3 to 158.1 ± 5.7 ms; $\tau_{f, in}$ from 17.4 ± 1.7 to 24.7 ± 14.0 ms, $n=7$, $p < 0.01$). However, DHA at $25 \mu M$, the concentration which dramatically suppressed H_2O_2 -induced EADs, did not show any significant effects on current amplitudes (peak $I_{T0} = 12.51 \pm 1.47$ pA/pF; $I_{T0, ss} = 3.34 \pm 0.31$ pA/pF, $n=6$, $p > 0.05$ compared to H_2O_2 , respectively) or inactivation process of I_{T0} ($\tau_{s, in}$: 154.6 ± 6.6 ms and $\tau_{f, in}$: 23.9 ± 1.1 ms, $n=7$, $p > 0.05$ compared to H_2O_2 ; Figures 4A–C). Furthermore, we found that H_2O_2 accelerated the recovery from inactivation of I_{T0} mainly by decreasing the fast component ($\tau_{f, re}$: from 817.2 ± 79.2 ms

to 341.9 ± 26.1 ms, $n=7$, $p < 0.05$), but not by changing the slow component ($\tau_{s, re}$: from control 5335.4 ± 504.8 ms to H_2O_2 4963.2 ± 459.9 ms, $p > 0.05$). Similarly DHA ($25 \mu M$) did not cause any significant alteration in I_{T0} recovery kinetics after H_2O_2 treatment (Figure 4D).

EFFECT OF DHA ON INTRACELLULAR ROS LEVELS

The level of oxidative stress may either increase or decrease in tissues from humans and animals supplemented with fish oil as reported previously (Garrido et al., 1989; Mas et al., 2010; Tsuduki et al., 2011). To determine whether DHA reduces the incidence of EAD via affecting (decreasing) intracellular ROS, the effect of DHA on intracellular ROS levels was measured in isolated ventricular myocytes treated with exogenous H_2O_2 ($200 \mu M$) by monitoring CM-DCF fluorescence intensity. The effect of DHA on intracellular ROS levels in the absence of H_2O_2 was also measured. As shown in Figure 5, exogenous H_2O_2 produced a rapid and dramatic increase in DCF fluorescence intensity in the myocytes and the F/F_0 of DCF fluorescence intensity reached a steady state value of 2.18 ± 0.24 at 6 – 10 min after H_2O_2 treatment. However, DHA ($25 \mu M$, either pretreatment or after treatment) showed no



significant effect on the fluorescence of CM-DCF either in the absence or presence of H_2O_2 (2.21 ± 0.33 , $n = 8$, $p > 0.05$).

DISCUSSION

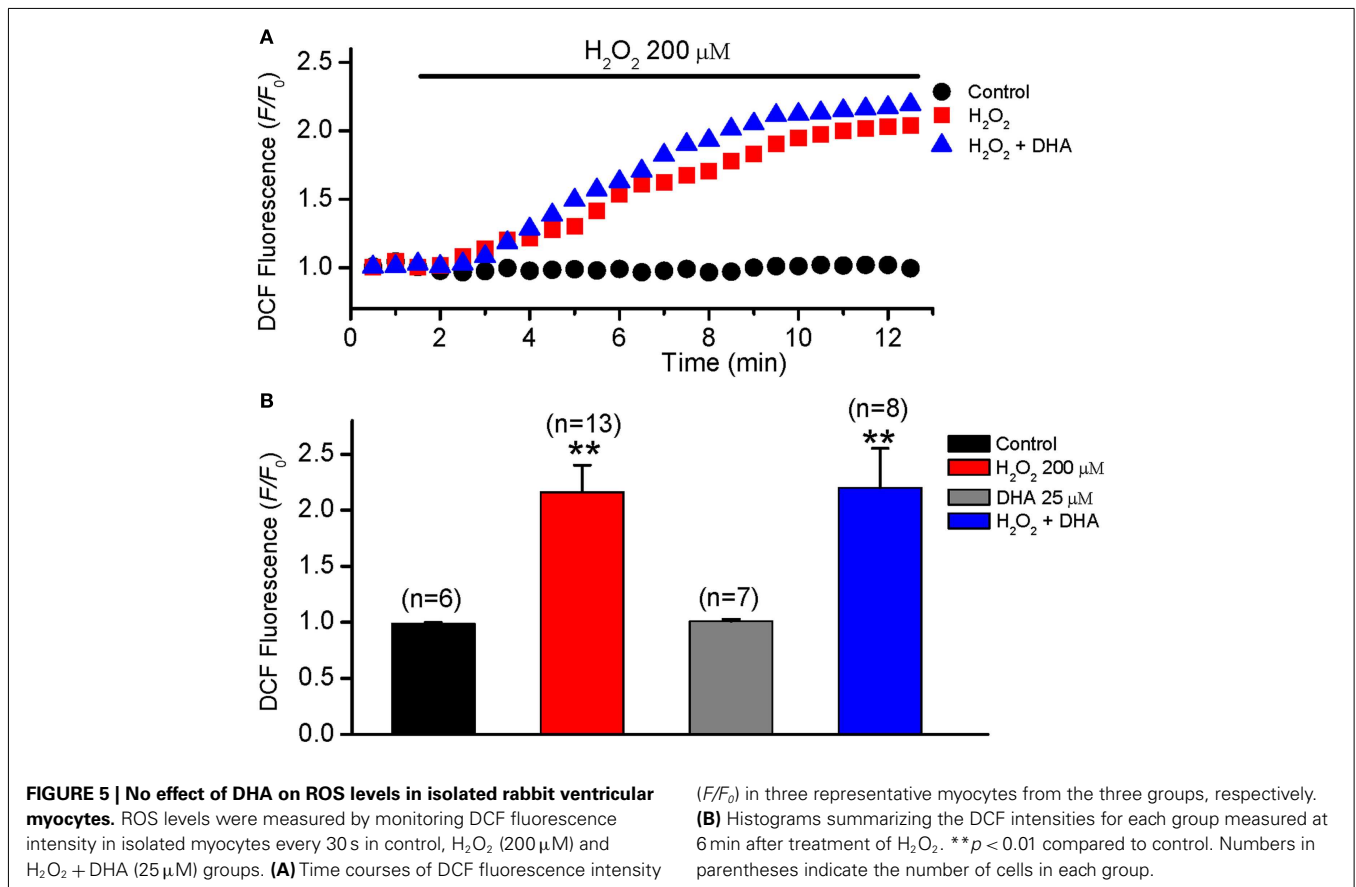
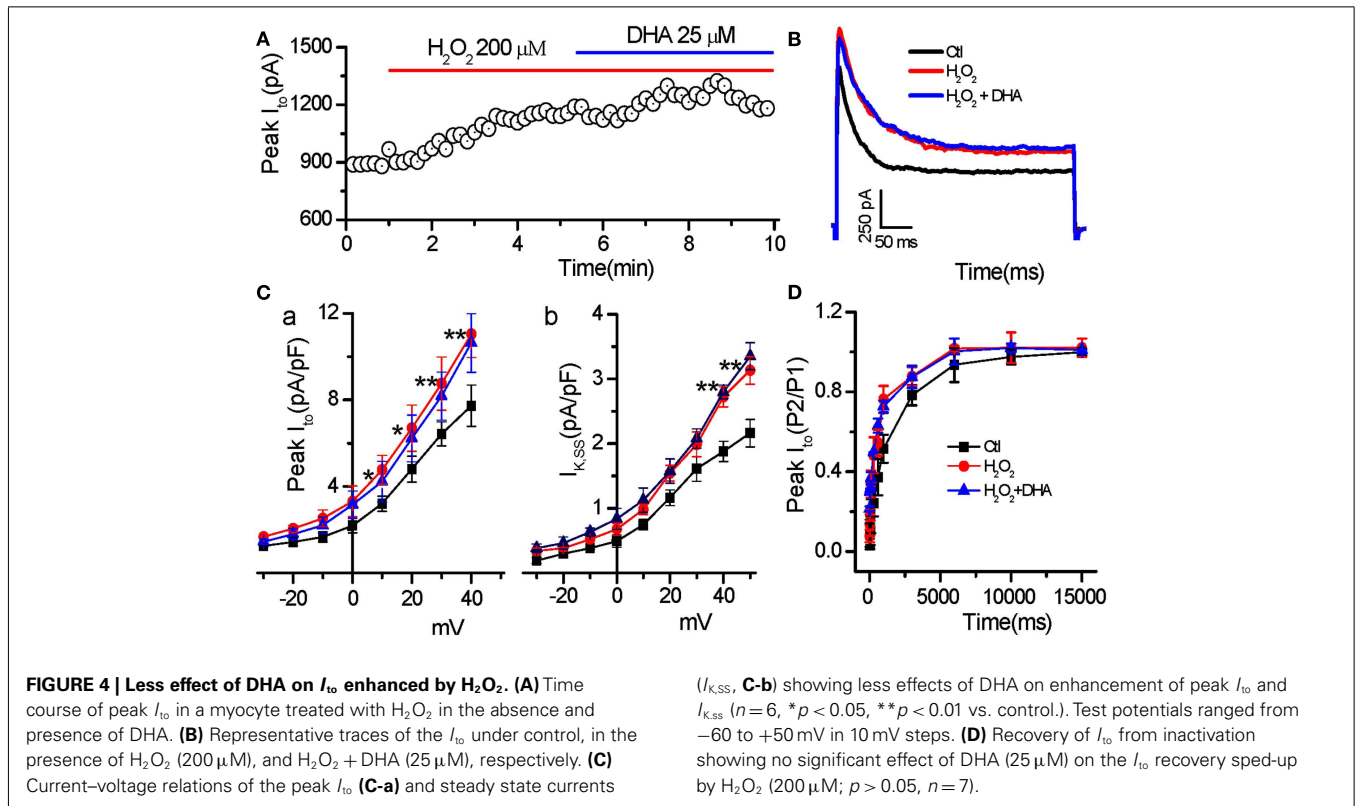
Experimental and clinical studies have obtained controversial results regarding the effects of fish oil or ω -3 PUFA on cardiac rhythm (von Schacky, 2008). Differences in the underlying pathogenic mechanisms for the arrhythmia in differing patient groups

or animal models may account for these controversies. We and others have previously shown that both exogenous and endogenous ROS-induced EADs can serve as triggers for arrhythmias. In the present study, we provide the first evidence showing that DHA attenuates EADs induced by H_2O_2 .

The molecular and ionic mechanisms of ion channel modulation by DHA are still not completely understood. A recent review article comprehensively summarized the potential antiarrhythmic electrophysiological effect of ω 3-PUFAs on the heart (Richardson et al., 2011). Inhibitory effects of DHA on EADs may involve multifactorial mechanisms e.g., (1) via ROS modulation. Although ω 3-PUFA may slightly increase levels of oxidative stress due to the susceptibility to oxidation, low to moderate ROS exposure can conversely give rise to up-regulation of antioxidant enzymes and increase antioxidant ability (scavenging ROS) in cardiac tissue (Jahangiri et al., 2006); (2) via direct modulation of ion channels by binding to the channels or affecting cell membrane lipid properties (such as membrane lipid peroxidation). While there is a widespread effect of ω 3-PUFA on ion channels and ion pumps, Ca^{2+} and Na^+ currents are most sensitive to ω 3-PUFAs (Richardson et al., 2011). Nevertheless, our present data suggest that the ionic mechanisms underlying inhibitory effect of DHA on EADs most likely involve the direct inhibition on the $I_{Ca,L}$ and late I_{Na} rather than its putative antioxidant ability. This notion was supported by the observation that there was no effect on CM-DCF fluorescence induced by DHA at the same concentration that led to reduction of EADs. In addition, the fast time course for DHA suppression of $I_{Ca,L}$ and late I_{Na} also supports a mechanism of direct inhibition of ion channels by DHA. Our most recent data showed H_2O_2 also activates I_{to} and may facilitate EAD generation (Zhao et al., 2012b). In the present study, however, we showed that DHA did not reverse the I_{to} activated by H_2O_2 in rabbit ventricular myocytes, which is inconsistent with previous reports that DHA markedly reduces I_{to} in human atrial cells and rat ventricular myocytes even at lower concentrations (5–10 μM ; Bogdanov et al., 1998; Verkerk et al., 2006; Li et al., 2009). We do not have a ready explanation for this discrepancy, while the molecular subtypes of I_{to} proteins might be different between rabbits and other species (including humans) or between different locations in the heart (e.g., ventricle vs. atria). In addition, the H_2O_2 -activated I_{to} seemed to be more resistant to DHA than the I_{to} at baseline, since we observed the inhibitory effects of 25 μM DHA on I_{to} (up to ~50%) in the absence of H_2O_2 .

It has also been reported that n-3-PUFAs are capable of reducing the activity of CaMKII (Zaloga et al., 2006), which may partially account for the inhibitory effect of DHA on EADs. However, since DHA does not alter the ROS level in the presence of H_2O_2 (Figure 5), the reduction of CaMKII activity, if any, may be mediated by less Ca entry secondarily to $I_{Ca,L}$ blockage, rather than by lower oxidation. Further experiments are needed identify the involvement of CaMKII.

Nevertheless, our present study suggests fish oil supplements may be effective in preventing/treating arrhythmias under an increased oxidative stress condition and serve as an alternative or complimentary anti-arrhythmic drug. Conditions with elevated oxidative stress level including ischemia/reperfusion, heart failure and aging might benefit from fish oil supplements.



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