

Non-enzymatic lipid mediators, neuroprostanes, exert the anti-arrhythmic properties of docosahexaenoic acid

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ABSTRACT

Neuroprostanes are lipid mediators produced by non-enzymatic free radical peroxidation of docosahexaenoic acid (DHA). It is associated to a lower atherosclerosis risk suggesting a beneficial role in cardiovascular diseases. The aim of this study was to investigate the influence of DHA peroxidation on its potentially anti-arrhythmic properties (AAP) in isolated ventricular cardiomyocytes and *in vivo* in post-myocardial infarcted (PMI) mice.

Calcium imaging and biochemical experiments indicate that cardiac arrhythmias induced by isoproterenol are associated with Ca^{2+} leak from the sarcoplasmic reticulum (SR) following oxidation and phosphorylation of the type 2 ryanodine receptor (RyR2) leading to dissociation of the FKBP12.6/RyR2 complex. Both oxidized DHA and 4(*RS*)-4- F_{4t} -NeuroP prevented cellular arrhythmias and posttranslational modifications of the RyR2 leading to a stabilized FKBP12.6/RyR2 complex. DHA *per se* did not have AAP. The AAP of 4(*RS*)-4- F_{4t} -NeuroP was also observed *in vivo*.

In this study, we challenged the paradigm that spontaneously formed oxygenated metabolites of lipids are undesirable as they are unconditionally toxic. This study reveals that the lipid mediator 4(*RS*)-4- F_{4t} -neuroprostane derived from non-enzymatic peroxidation of docosahexaenoic acid, can counteract such deleterious effects through cardiac anti-arrhythmic properties.

Our findings demonstrate 4(*RS*)-4- F_{4t} -NeuroP as a mediator of the cardioprotective AAP characteristics of DHA. This discovery opens new perspectives for products of non-enzymatic oxidized omega-3 polyunsaturated fatty acids as potent mediators in diseases that involve ryanodine complex destabilization such as ischemic event.

Keywords:

Oxidative stress, Neuroprostanes, anti-arrhythmic, cardioprotection, DHA, Ryanodine receptor, calcium

Abbreviations:

4(*RS*)-4- F_{4t} -NeuroP, 4(*RS*)-4- F_{4t} -neuropropane; ω 3 PUFAs, omega-3 polyunsaturated fatty acids; AAP, anti-arrhythmic properties; AoVTI, aortic velocity time integral; COX-2, cyclooxygenase; CYP450, cytochrome P450; DHA, docosahexaenoic acid; DNP, carbonylated RyR; ECG, electrocardiograms; EF, ejection fraction; EPA, eicosapentaenoic acid; ES, extrasystoles; F, fluorescence; FAC, fractional area change; FS, fraction shortening; FsK, forskolin; GPx, glutathione peroxidase enzymes; H_2O_2 , hydrogen peroxide; HR, heart rate; ISO, isoproterenol; LOX, lipoxygenase; LV, left ventricular; NE, norepinephrine; PMI, post-myocardial infarcted; QTc, corrected QT interval; ROS, reactive oxygen specie; RyR 2, type 2 ryanodine receptor; RYR2-p2808, Phosphorylated RyR PKA site serine 2808 ; SCD, sudden cardiac death; sHE, epoxide hydrolase (diols); S-NO, S-Nitrosylated RyR; SR, sarcoplasmic reticulum; Vit E, vitamin E

INTRODUCTION

The cardioprotective effects of omega-3 polyunsaturated fatty acids (ω 3 PUFAs) have been evident since the mid-70s [1]. The intake of fatty fish such as mackerel or tuna is associated with a lower risk of cardiac arrhythmias including sudden cardiac death (SCD) [2–4] and arrhythmic coronary heart disease death [5]. Administration of Omacor[®], a mixture of 850 mg of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) the two major polyunsaturated fatty acids (PUFAs) of fatty fish, decreased the incidence of SCD in secondary prevention of myocardial infarction [6]. The mechanisms responsible for the anti-arrhythmic properties (AAP) of PUFAs remain unclear. In dogs, infusion of a DHA emulsion tended to slow heart rate (HR), shortened the corrected QT interval (QTc) at rest and significantly prevented ischemia-induced fatal ventricular arrhythmias [7]. These experiments confirmed previous reports on the prevention of ischemia-induced ventricular arrhythmias in dogs [8] and marmosets [9] by PUFAs. In humans, a significant slowing of HR and the likelihood of prolonged QT has been observed [10].

Experimental studies on isolated cardiac cells suggest that ω 3 PUFAs have direct cardiac electrophysiological effects [11]. However, DHA is highly prone to peroxidation and we have shown that non-enzymatic oxygenated products of DHA and not DHA *per se* are active on cardiac ionic channels [12]. In agreement, it has been demonstrated in rabbit ventricular cells that early depolarization induced by H₂O₂ is inhibited by DHA while reactive oxygen species (ROS) production is not altered, indicating the resiliency of oxidized DHA [13].

DHA can be oxidized through two pathways; enzymatically, resulting in the production of compounds such as resolvins or maresins, or non-enzymatically by ROS initiation and propagation of free radical reactions, leading to the release of numerous products, including neuroprostanes (F₄-NeuroPs) [14]. Neuroprostanes are recognized as oxidative stress biomarkers for the DHA-rich brain and are associated to ischemic stroke and neurodegenerative diseases [15,16]. More recently, it has been proposed that F₄-NeuroPs may play a favorable role as potential bioactive components in identifying atherosclerosis risk [17].

In the present study, we show through *in cellulo* and *in vivo* approaches that non-enzymatic oxidation of DHA is a prerequisite to obtain ventricular anti-arrhythmic effects. In particular, one of the F₄-NeuroPs isomer, 4(*RS*)-4-F_{4t}-neuroprostane (4(*RS*)-4-F_{4t}-NeuroP)

appears to be the main anti-arrhythmic metabolite of DHA in preventing deleterious post-translational modification of RyR2 and thus regulating calcium homeostasis.

MATERIALS AND METHODS

Animal experiments

Male C57Bl/6 mice (Janvier, France) of 7 weeks old were randomly assigned into two main groups 1) mice with post-myocardial infarction (PMI mice) after left coronary artery ligation as previously described [18] and 2) sham-operated mice that were submitted to the surgical procedure but not to the artery ligation. All animal-handling procedures conformed to European Parliament Directive 2010/63/EU and the institutional animal research committee council on the protection of animals (CEEA-LR-12096).

In brief, anesthesia was performed for left thoracotomy and cardiac monitoring (2% isoflurane/O₂, Aerrane®, Baxter, France). The artery was ligated 1-2 mm beyond the emergence from the top of the left atrium, using an 8-0 suture for PMI mice. A subcutaneous injection of 0.01 mL buprenorphine solution (0.3 mg/mL) for post-operative analgesia was administered. Shams were subjected to the same surgical procedure but without coronary artery ligation. The mice were housed in single cages in a room under regulated temperature and hygrosopic conditions (23±1°C, 45±10% humidity, light-dark schedule of 12h:12h *ad libitum* feed).

After 4 weeks, the mice were randomly assigned to the different treatment groups: Sham and PMI dosed with vehicle (NaCl 0,9%); PMI mice treated with 10 µM DHA (PMI DHA); PMI treated with 10 µM DHA and 1 µM α -tocopherol (PMI DHA + Vit E); PMI mice treated with 10 µM DHA and 1 µM hydrogen peroxide (PMI DHA + H₂O₂) and PMI mice treated with 1 µM 4(RS)-4-F₄-NeuroP. We chose to work on PMI mice challenged with NE since it has been shown that the AAP of DHA is secondary to myocardial infarction in human [6].

Treatments were administrated as intravenous injection (200 µl) of the prepared solution equivalent to 10 times the concentration to reach the final concentrations matching *in cellulo* experiments.

It is known that the activation of the adrenergic nervous system is one factor that may play a crucial role in the genesis of arrhythmias associated with acute myocardial infarction [19]. To mimic this activation, all PMI mice were then intra-peritoneally (i.p.) challenged

with the β 1-adrenergic agonist, norepinephrine (2.5 mg/kg) [20] 20 min after they received their treatment.

Echocardiography

Doppler echocardiography was performed using a high-resolution ultrasound system (Vevo 2100; VisualSonics, Toronto, Canada) equipped with a 40-MHz transducer. The mice were anesthetized with 1.5% isoflurane in 100% oxygen and placed on a heating table in a supine position. Body temperature was monitored through a rectal thermometer to be maintained at 36-38°C and electrocardiograms (ECG) were recorded all along the echocardiographic procedure with limb electrodes. Ejection (EF%) and shortening (SF%) fractions were calculated from the left ventricular diameters on M-mode measurements at the level of papillary muscles in a parasternal short-axis two-dimensional view. To better consider coronary ligation-induced left ventricular remodeling, EF was also calculated from a B-mode parasternal long axis view (EF% B-mode) by tracing endocardial end-diastolic and end-systolic areas to estimate left ventricular volumes, and the endocardial fractional area change (FAC%) on a parasternal short-axis view at papillary muscle level was calculated. Pulsed-wave Doppler of the ascending aortic blood flow was recorded permitting measurements of the velocity time integral (AoVTI). All measurements were quantified and averaged for three cardiac cycles (Table 1).

Synthesis of 4(RS)-4-F_{4t}-NeuroP

Using the protocol previously reported, we synthesized F_{4t}-NeuroPs. The strategy is based on an easily accessible bicyclic precursor to obtain isoprostanoid derivatives [21], while more refined strategy were used for the synthesis of the isomers, 4(RS)-4-F_{4t}-NeuroP [22], 10-F_{4t}-NeuroP [23] and 14(RS)-14-F_{4t}-NeuroP (non yet published). The 13-F_{4t}-NeuroP was synthesized using another strategy [24].

Fatty acid solution and oxidation

To observe antioxidant or oxidant effect, cells were incubated 20 minutes in Tyrode solution containing in 10 μ M DHA or 10 μ M DHA + 1 μ M Vit E or 10 μ M DHA + 1 μ M H₂O₂. To prepare these solutions, DHA (stock prepared in ethanol) was added in the Tyrode solution after Vit E (stock prepared in chloroform) or H₂O₂ (stock prepared in reverse osmosis water). Stock solution of 4(RS)-4-F_{4t}-NeuroP was prepared in Tyrode solution and diluted accordingly for the experimentations.

Quantification of 4(RS)-4-F_{4t}-NeuroP

In Tyrode solution (control), 10 μ M DHA was incubated with or without 1 μ M α -tocopherol (Vit E) or 1 μ M hydrogen peroxide (H₂O₂) for 20 min in room temperature. The reaction was terminated with antioxidant butylated hydroxytoluene BHT (0.005%, w/v). The internal standard C21-15-F_{2t}-IsoP (2.5 ng), synthesized by IBMM (Montpellier, France) laboratory was added to each sample mix. The sample was further diluted in aqueous sodium acetate solution (pH 4.6), acidified with 1M HCl and applied to pre-washed (methanol) Bond Elut Certify II SPE cartridge (Agilent, CA USA). After loading the sample (control, DHA or DHA+VitE or DHA+H₂O₂), it was sequentially cleaned with water/methanol (1:1) and hexane / ethyl acetate (7:3) and then F₄-NeuroPs was eluted with ethyl acetate/methanol (9:1). The eluate was dried under nitrogen and then derivatized in room temperature for 30 min with 10% pentafluorobenzyl bromide and 10% N,N-diisopropylethylamine prepared in acetonitrile (2:1). Thereafter, it was dried under nitrogen and then derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + trimethylsilylchlorosilane (TMCS) 1% and N,N-dimethylformamide (2:1) (Sigma Aldrich, USA). After drying the reagents under nitrogen, samples were re-suspended in decane.

Gas chromatography-mass spectrometry set at negative ion chemical ionization (TraceGC and DSQ II Mass Spectrometer, Thermo Fisher Scientific, MA USA) was used to determine 4(RS)-4-F_{4t}-NeuroP [25]. Analytical column FactorFour™ (Varian, USA) fused silica capillary was used. Helium gas was the carrier gas and the column temperature was programmed from 140°C to 250°C at 30°C per minute then 250°C to 300°C at 4°C per minute and remained at this temperature for 10 minutes. The ion source temperature was 200°C and isobutane (1 mL/min) was used as the reagent gas for NICI. Selected ion monitoring was performed to monitor ions m/z 593.5 for 4(RS)-4-F_{4t}-NeuroP and at m/z 583.5 for C21-15-F_{2t}-IsoP internal standard. Quantitation was achieved by relating the peak area of the 4(RS)-4-F_{4t}-NeuroP with C21-15-F_{2t}-IsoP internal standard peak.

Preparation of cardiomyocytes

Cellular experiments were performed on freshly isolated left ventricular myocytes from the non-infarcted free wall (excluding the border zone). In brief, after cervical dislocation, the heart was removed, washed and aorta was cannulated to a modified Langendorff system. The heart was perfused by retrograde flow rate of 5–10 mL/min at 37°C for 6–8 min with a modified Tyrode solution composed of 113 mM NaCl, 4.7 mM, KCl, 0.6 mM KH₂PO₄, 0.6

mM Na₂HPO₄, 1.2 mM MgSO₄, 12 mM NaHCO₃, 10 mM KHCO₃, 10 mM Hepes, (pH 7.4) and 0.1 g/mL liberase dispase (high research grade, Roche, France).

After enzymatic treatment (4-6 min), a part of the left ventricle was removed and minced to separate the cells. Isolated myocytes were re-suspended in a sterile enzyme-free Tyrode solution, and the Ca²⁺ concentration of the ventricular cell suspension was gradually increased to 1 mM by the addition of CaCl₂ in five sequential steps 100, 100, 300 and 500 μM with 10 min interval between steps. Finally the cardiomyocytes were kept at room temperature 22 – 24° C until use. Prior to the treatments, the freshly isolated cardiomyocytes were then superfused with standard Tyrode solution (121 mM NaCl, 5.0 mM KCl, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 0.4 mM NaH₂PO₄, 24 mM NaHCO₃, 0.1 mM EDTA and 5.5 mM glucose). Cardiomyocytes with obvious sarcolemmal blebs or spontaneous contractions were not used. Only cardiomyocytes with clear edges were selected and were used within 1-6 h after isolation.

Inhibition of enzymatic lipid peroxidation of cardiomyocytes

Inhibitors of enzymatic lipid peroxidation, anti-lipoxygenase (1 μM zileuton), anti-cytochrome P450 (3 μM ketoconazole) and anti-cyclooxygenase 2 (1 μM celecoxib) from Sigma-Aldrich, USA and anti-epoxide hydrolase (10 nM) from Cayman Chemicals USA, and a combination of 4 inhibitors were tested with and without 10 μM DHA + 1 μM H₂O₂ in the cellular arrhythmias. We also used glutathione peroxidase enzyme, which reduce lipid peroxides to alcohols and H₂O₂. Stock solution of GPx was dissolved in water with 10 mM of phosphate sodium and 1 mM of dithiothreitol. GPx was either added before the mix DHA + H₂O₂ at concentration of 10 units (1 unit oxidize 1 μM of DHA per minute) in Tyrode solution or after the DHA + H₂O₂ mix. For all the experiments, solutions were prepared freshly from the stock and diluted with Tyrode medium.

Calcium channeling

The effect of oxidation of ω-3 PUFAs on cell shortening and Ca²⁺ transients of field-stimulated cardiomyocytes is monitored online using commercial myocyte calcium and contractility monitoring system (IonOptix®, Milton, MA, USA) connected to a standard inverted fluorescent microscope. Cells were field-stimulated with 1 ms current pulses delivered via two platinum electrodes. To monitor intracellular Ca²⁺ concentration, cardiomyocytes were loaded with the fluorescent ratiometric Ca²⁺ indicator Indo-1AM (2 μM, Invitrogen, France). They were simultaneously illuminated at 365 nm using a xenon arc bulb

light. Cytosolic Ca^{2+} concentration was determined by Indo-1 AM fluorescence which emit at 405 nm and 480 nm concurrently. The ratio of 405nm/480nm indicates the cytosolic Ca^{2+} concentration.

To observe arrhythmias (ventricular extrasystoles), the cells were bathed with 10 nM isoproterenol and stimulated with 30 s pacing period (1.0 Hz), followed by 30 s rest period [20,26]. Confocal imaging was performed using a Zeiss LSM510 confocal microscope (Carl Zeiss Inc., Oberkochen, Germany) equipped with an argon laser (488 nm) and a 60X, 1.3 NA oil immersion objective set at axial and radial resolutions of 1.0 and 0.4 μm , respectively.

Ca^{2+} sparks were recorded in quiescent myocytes incubated with the Ca^{2+} indicator Fluo-4-AM (4 μM) (Molecular Probes, OR USA) for 15 min. The dye was excited at 488 nm and the fluorescence emission was collected through a 505 nm long-pass filter. Myocytes were field-stimulated at 1 Hz with 1 ms current pulses delivered via two platinum electrodes, one on each side of the perfusion chamber. During the rest period that follows stimulation, myocyte were repetitively scanned along the entire length of the cell at 1.5 ms intervals, for a maximum of 6 s. The laser intensity was reduced to 5% maximum to decrease cell damage and dye bleaching. Line scan diagrams were constructed by stacking emission lines, corresponding to excitation scans, in temporal order. An average of the Ca^{2+} sparks were determined by the intensity of each sequential scan line and plotting the mean intensity as a function of time. The SparkMaster® plugin for ImageJ® software was used to detect and analyze Ca^{2+} sparks.

Immunoblot

Proteins were extracted from basal left ventricular frozen cells (50 mg) homogenized with a manual polytron® instrument. Cells were then lysed in 600 μL extraction buffer (Tris maleate 10m M, NaF 35 mM, Triton 1%, activated orthovanadate 20 mM, inhibitor cocktail, Roche, France) for 45 min under rotated agitation. Membrane and cytosolic proteins were collected from the supernatant after 5 min centrifugation at 10, 000 x g at 4 °C.

For the immunoprecipitation assay, left ventricular (LV) tissues were lysed in 1 mL buffer containing 10 mM Tris maleate (pH 6.8), 35 mM NaF, triton 1% and protease inhibitors (Roche 11873580001, France). A concentration of 10 μg anti-RyR2 antibody was used to immunoprecipitate RyR2 from 500 μg of LV homogenate. The samples were incubated with anti-RyR antibody in 0.5 ml modified RIPA buffer (Tris HCl 10 mM, pH 7.4, NaCl 150 mM, Triton 1%, NaF 5 mM and protease inhibitor cocktail) for 2 hours at 4°C. The

immune complexes were incubated with protein A/G magnetic beads (Pierce 88802, USA) at 4°C for 2 hours, after which the beads were washed three times with RIPA buffer.

To detect RyR2 protein oxidation, the immune complex was treated with 2,4 dinitrophenylhydrazine (DNPH) and the DNP-derivatized protein samples formed were detected using on Immunoblot Protein oxidation detection Kit (Millipore S7150, USA). Proteins were then separated using SDS-PAGE, blotted onto nitrocellulose membranes (0.2 µm, GE Healthcare, France) and incubated overnight at 4°C with primary antibodies: the type 2 ryanodine receptor, RyR2 (1/1000 dilution, Pierce, France) and anti FKBP12.6 (1/1000 dilution, RD System AF 4174, France). Protein levels were expressed relative to GAPDH (1/60000 dilution, AB8245, ABCAM, France). All immunoblots were developed and quantified using the Odyssey infrared imaging system (LI-COR Biosystems, USA) using infrared-labeled anti-mouse and anti-rabbit IgG (1/30000 dilution) secondary antibodies.

Statistical analysis

All data are given as mean ± SEM. Statistical analyses were performed using GraphPad Prism® (Prism 5 for Mac OS X). One-way ANOVA for multiple comparisons was used, followed by a parametric t-test with Fishers correction. For paired studies, Wilcoxon signed rank test is used. Percentage of arrhythmic cells data was analyzed by a χ^2 -test. A p-value of 0.05 or less was indicated as statistically significant.

RESULTS

DHA under oxidative stress conditions reduced cardiac arrhythmias

AAP of DHA was evaluated in models of cardiac arrhythmias. Different conditions of oxidative status were obtained by preparing DHA solutions in the presence of an antioxidant, 1 μ M α -tocopherol [27] or a pro-oxidant, 1 μ M H_2O_2 [28]. Bathing isolated mice ventricular cardiomyocytes for 20 min in DHA prevented arrhythmias and concurs in part with previous report [11]. Importantly, AAP of DHA was potentiated by pro-oxidants and conversely prevented in the presence of α -tocopherol (Figures 1A and 1B). When applied alone, H_2O_2 and α -tocopherol had no effect (Figure 1B), which further support the role of DHA oxidation on AAP.

Additionally, we investigated the efficacy of DHA to reduce the trigger of ventricular extrasystoles (ES) in a post myocardial infarction (PMI) mice model established by coronary artery ligation and sensitized by norepinephrine (NE) [29]. PMI mice develop calcium dependent-ES due to increased diastolic calcium level in a context of increased ROS production [30]. These arrhythmias are potentiated by a norepinephrine challenge. In this validated model and as described in Table 1, intravenous injection of DHA to the mice reduced ES by 45%, which further reinforced the AAP of DHA in pro-oxidant conditions (Figures 1C and 1D).

AAP are not mediated by enzymatic oxidation of DHA

Enzymatic oxidation of DHA can develop endogenously [31], like the conditions used in this experimental approach. The AAP of DHA was investigated in cardiomyocytes in the presence of different inhibitors of enzymes that oxidize PUFAs namely cyclooxygenase (COX-2), lipoxygenase (LOX) and cytochrome P450 (CYP450). Individually or combined, the inhibitors did not modify the AAP of pro-oxidized DHA (Figure 2A). Our observation infers that DHA exert a strong AAP through non-enzymatic peroxidation process that generates metabolites such as F_4 -neuroprostanes and not through typical enzymatic process that involves for example resolvins, protectins and maresins. In order to further explore the chemical entities needed for the observed AAP, we buffered any hydroperoxyl derivatives potentially formed by our pro-oxidant condition (DHA+ H_2O_2) by a late addition of glutathione peroxidase enzymes (GPx) (Figure 2B). AAP were still observed in these

conditions, indicating these effects are not related to endoperoxide or hydroperoxide metabolites of DHA. On the contrary, arrhythmia persisted when GPX was added prior to DHA+H₂O₂ indicating that H₂O₂ initiated the formation of the required metabolites of DHA for AAP (Figure 2B).

Among the F₄-neuroprostanes, 4(RS)-4-F_{4t}-NeuroP had the most active AAP

Supplementation of DHA to atherosclerotic LDLR^{-/-} mice showed that liver F₄-NeuroP concentration is negatively correlated to atherosclerosis risk [17]. The isomer 4(RS)-4-F_{4t}-NeuroP is the most abundant F₄-NeuroPs formed from non-enzymatic DHA peroxidation [14]. In our *in vitro* experiments, incubation of DHA (10 μM) with H₂O₂ (1 μM) in Tyrode solution for 20 min generated 0.61±0.08 μM of 4(RS)-4-F_{4t}-NeuroP (Figures 3A and B) whereas DHA with Vit E had no 4(RS)-4-F_{4t}-NeuroP formed and DHA alone had trace amount (0.03±0.01 μM). Furthermore, we recently discovered that levels of 4(RS)-4-F_{4t}-NeuroP and another isomer, 10-F_{4t}-neuropropane (10-F_{4t}-NeuroP) are concentrated in brains of preterm pigs [32], and in adult rat brain and heart [33].

From our findings, the high concentration of 4(RS)-4-F_{4t}-NeuroP in the heart indicates a potential bioactive role. We compared the anti-arrhythmic effect of 4(RS)-4-F_{4t}-NeuroP with other F₄-neuroprostanes (10-F_{4t}-NeuroP, 13-F_{4t}-NeuroP, 14(RS)-4-F_{4t}-NeuroP) (Table 2). Of the four F₄-neuroprostanes tested, the 4(RS)-4-F_{4t}-NeuroP is the most potent (IC₅₀≈100 nM) (Figure 3C).

Despite the IC₅₀≈100nM of 4(RS)-4-F_{4t}-NeuroP, we subjected the maximum concentration (1 μM) to authenticate the AAP in our study. Our *in vivo* evaluation indicates AAP of 1 μM 4(RS)-4-F_{4t}-NeuroP was comparable with the positive control 1 μM carvedilol, which is a referenced anti-arrhythmic drug (Figure 3D). Also, 4(RS)-4-F_{4t}-NeuroP inhibited arrhythmias produced by the adenylyl cyclase activator, forskolin (Figure 3E) suggesting that β-blocking properties is not involved in the AAP of 4(RS)-4-F_{4t}-NeuroP. Absence of bradycardia following 4(RS)-4-F_{4t}-NeuroP consolidates this hypothesis (Table 1). Further, the AAP of DHA+H₂O₂ and 4(RS)-4-F_{4t}-NeuroP were similar suggesting that the AAP of DHA is largely due to the generation of 4(RS)-4-F_{4t}-NeuroP in DHA+H₂O₂.

4(RS)-4-F_{4t}-NeuroP prevents RyR2 dysfunction

In our experimental models, the arrhythmias prevented by 4(*RS*)-4- F_{4t} -NeuroP are mainly due to extrasystoles (ES). The mechanisms of triggering ES include alterations of Ca^{2+} homeostasis that are potentiated by catecholamines; the two phenomena observed after myocardial infarction [34]. The Ca^{2+} signaling alteration could be associated with a leaky type 2 ryanodine receptor (RyR2) [35–37]. As a consequence, cytosolic Ca^{2+} levels in diastole rise and promote ES [38]. We thus evaluated the leaky behavior of RyR2 by measuring the frequency of spontaneous Ca^{2+} sparks by confocal microscopy [39] and the resting cytosolic Ca^{2+} concentration by epifluorescence. In our model, Ca^{2+} sparks events frequency was increased by isoprenaline (Figures 4A and 4B). This increase was partially prevented by DHA as also shown in previous reports [40,41]. Interestingly, the effects of DHA on Ca^{2+} sparks frequency were blunted by α -tocopherol and enhanced by H_2O_2 . PMI cardiomyocytes also exhibited an increased frequency of Ca^{2+} sparks that were significantly reduced by 4(*RS*)-4- F_{4t} -NeuroP. The changes in the sparks frequency followed changes in resting Ca^{2+} concentration (Figure 4C).

At the molecular level, post-translational modifications of RyR2 may account for their leaky behavior [35–37]. We quantified RyR2 carbonylation, S-nitrosylation, phosphorylation and the degree of association of RyR2 with FKBP12.6 on the arrhythmic cardiomyocytes after 10 nM isoproterenol (ISO) treatment with or without 10 μ M DHA, 10 μ M DHA + 1 μ M Vit E or 10 μ M DHA + 1 μ M H_2O_2 (Figure 5). Iso challenge promotes dissociation of FKBP12.6 from the RyR2 macromolecular complex as previously reported and accounts for the abnormal SR- Ca^{2+} leak [35] (Figure 5A). Iso also promotes RyR2 carbonylation, S-nitrosylation and phosphorylation on serine 2808 (Figure 5B-D). These effects were prevented by DHA and more so by DHA + H_2O_2 compared to Iso and DHA + Vit E. This is supported by the lack of FKBP12.6 dissociation from the RyR2 complex of DHA or DHA + H_2O_2 treatment compared to Iso and DHA + Vit E (Figure 5A). Additionally, our observations indicate that oxidation of DHA is necessary to prevent RyR2 modification as no effect was found in the presence of Vit E, an antioxidant. In order to verify that the involvement of such an effect is due to a non-enzymatic oxidized lipid product of DHA, we repeated the evaluation on the arrhythmic cardiomyocytes with 4(*RS*)-4- F_{4t} -NeuroP (Figure 5A and 5B). The prevention of RyR2 phosphorylation and dissociation of FKBP12.6 of NeuroP was comparable to DHA + H_2O_2 . This strongly supports that the effects of oxidized DHA and 4(*RS*)-4- F_{4t} -NeuroP in correcting SR Ca^{2+} leak, normalizing diastolic Ca^{2+} level and preventing ES triggering results from the counteraction of RyR2 post-translational modifications and RyR2 dysfunction.

DISCUSSION

In the present study, we clearly demonstrated that oxidized DHA possesses potent AAP *in cellulo* and *in vivo*, and that unmodified DHA *per se* was inactive. This effect is not mediated by enzymatic lipid peroxidation but instead by neuroprostanes, the stable end products of the non-enzymatic lipid peroxidation of DHA, particularly 4(*RS*)-4-F_{4t}-NeuroP. This metabolite displayed a unique and unprecedented mode of action in the family of lipid mediators and of any known endogenous biomolecule, as it stabilized RyR2 and maintained this complex closed during diastole.

This discovery bridges the missing relationship between cardiac ischemic events and the AAP of DHA. Indeed, following cardiac ischemic diseases and myocardial infarction there is a burst of ROS [42] that can be involved in the generation of the arrhythmias, the origin of SCD [43]. Such an abrupt imbalance of the oxidative status can oxidize proteins [44], nucleic acids [45] and lipids [46]. These highly reactive compounds play a pivotal role in the pathogenesis of post-ischemic injury that progresses to SCD [47,48]. Classically, there is a consensus that overproduction of ROS are mainly deleterious and do not play a role in normal physiology. For example it has been shown that an isoketal, E₂-IsoK, originating from the oxidation of arachidonic acid can produce adducts with the sodium channel protein Nav1.5, perturbing its activity in a pro-arrhythmic way [47]. However, the possibility that non-enzymatic oxygenated metabolites of ω3 PUFAs can exert counter effects has been under investigated.

We have previously reported that DHA needs to be oxidized to influence ionic channel activities [12] and it is agreed by others that such effects on ionic channels contributes to the AAP of DHA [49]. We hereby demonstrated that the AAP of DHA are dependent on the oxidative status of the environment, and that non-enzymatic auto-oxidation of DHA was a prerequisite to AAP. Our assays pinpointed neuroprostane metabolites as the potential metabolites with AAP properties. The main isomer, 4(*RS*)-4-F_{4t}-NeuroP, showed potent AAP *in cellulo* in a dose dependent manner and also *in vivo* in PMI mice.

At the cellular level, the mechanism of action is unlikely to be due to a β-blocker effect, but the AAP can instead be explained by a rycal-like effect, in particular, stabilization of the RyR2 complex with FKBP12.6 [50]. This effect was strongly supported by both oxidized DHA and 4(*RS*)-4-F_{4t}-NeuroP in correcting SR Ca²⁺ leak, normalizing diastolic Ca²⁺ levels and from the prevention of the dissociation of FKBP12.6 from RyR2. Although we can not exclude a direct effect of 4(*RS*)-4-F_{4t}-NeuroP on RyR2, in contrast to rycal effects, we

also observed that both oxidized DHA and 4(*RS*)-4-F_{4t}-NeuroP prevent RyR2 hyperactive S-nitrosylation, oxidation and phosphorylation, suggesting an upstream mechanism rather than a direct effect on RyR2 as previously reported [37]. Altogether we showed that 4(*RS*)-4-F_{4t}-NeuroP is capable of preventing ES triggering effects from the prevention of RyR2 post-translational modifications and RyR2 dysfunction by normalizing RyR2 activity in diastole and thus calcium homeostasis.

Emulsion of ω 3 PUFAs, injected intravenously has been shown to be anti-arrhythmic in a canine model of myocardial infarction [7] and similar effects on sustained ventricular tachycardia have been observed in humans [20,51]. The use of an *in vivo* ischemic model to monitor the triggering of ventricular ES clearly confirmed our hypothesis that DHA auto-oxidatively has to be transformed into neuroprostane for rapid inhibition of arrhythmias.

The pre-requisite for DHA oxidation to elicit any AAP could explain the lack of elucidation for the beneficial effects of ω 3 PUFAs other than in ischemic cardiovascular diseases [2,52,53]. After initial reports establishing that ω 3 PUFAs have cardioprotective properties, the beneficial effects were then extended to many if not all cardiovascular problems, even those that are not ischemic [53,54]. This mindset could be the drawback for the proper development of therapeutic targets in cardiac ischemia and for the difficulty in justifying its valuable impact in cardioprotection. Overall, the mechanism of action we describe here can explain the favorable impact of ω 3 PUFAs in chronic diseases implicating RyR2 dysfunction when an unbalanced oxidative status is present.

CONCLUSION

From this work, we conclude that the oxidation of DHA to 4(*RS*)-4-F_{4t}-NeuroP is necessary to prevent ischemia-induced arrhythmias. We propose that in oxidative stress conditions such as ischemic diseases, non-enzymatic oxygenated metabolites of DHA formed by peroxidation of cardiac membrane lipids, notably 4(*RS*)-4-F_{4t}-NeuroP, are responsible for the AAP of DHA by countering the cellular stress by ROS. Importantly, it appears that non-enzymatically oxygenated metabolites of ω 3 PUFAs can communicate and exert a physiological role. This highlights potential beneficial effects of increased ROS production dependent on the cellular environment.

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REFERENCES

- [1] Bang, H. O.; Dyerberg, J.; Hj orne, N. The composition of food consumed by Greenland Eskimos. *Acta Med. Scand.* 200: 69–73; 1976.
- [2] Burr, M. L.; Fehily, A. M.; Gilbert, J. F.; Rogers, S.; Holliday, R. M.; Sweetnam, P. M.; Elwood, P. C.; Deadman, N. M. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). *Lancet* 2: 757–761; 1989.
- [3] Siscovick, D. S.; Raghunathan, T. E.; King, I.; Weinmann, S.; Wicklund, K. G.; Albright, J.; Bovbjerg, V.; Arbogast, P.; Smith, H.; Kushi, L. H. Dietary intake and cell membrane levels of long-chain n-3 polyunsaturated fatty acids and the risk of primary cardiac arrest. *JAMA* 274: 1363–1367; 1995.
- [4] Albert, C. M.; Campos, H.; Stampfer, M. J.; Ridker, P. M.; Manson, J. E.; Willett, W. C.; Ma, J. Blood levels of long-chain n-3 fatty acids and the risk of sudden death. *N. Engl. J. Med.* 346: 1113–1118; 2002.
- [5] Mozaffarian, D.; Lemaitre, R. N.; Kuller, L. H.; Burke, G. L.; Tracy, R. P.; Siscovick, D. S.; Cardiovascular Health Study. Cardiac benefits of fish consumption may depend on the type of fish meal consumed: the Cardiovascular Health Study. *Circulation* 107: 1372–1377; 2003.
- [6] Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. *Lancet* 354: 447–455; 1999.
- [7] Billman, G. E.; Kang, J. X.; Leaf, A. Prevention of sudden cardiac death by dietary pure omega-3 polyunsaturated fatty acids in dogs. *Circulation* 99: 2452–2457; 1999.
- [8] Billman, G. E.; Hallaq, H.; Leaf, A. Prevention of ischemia-induced ventricular fibrillation by omega 3 fatty acids. *Proc. Natl. Acad. Sci. U. S. A.* 91: 4427–4430; 1994.
- [9] McLennan, P. L.; Bridle, T. M.; Abeywardena, M. Y.; Charnock, J. S. Dietary lipid modulation of ventricular fibrillation threshold in the marmoset monkey. *Am. Heart J.* 123: 1555–1561; 1992.
- [10] Mozaffarian, D.; Prineas, R. J.; Stein, P. K.; Siscovick, D. S. Dietary fish and n-3 fatty acid intake and cardiac electrocardiographic parameters in humans. *J. Am. Coll. Cardiol.* 48: 478–484; 2006.
- [11] Leaf, A.; Kang, J. X.; Xiao, Y.-F. Fish oil fatty acids as cardiovascular drugs. *Curr. Vasc. Pharmacol.* 6: 1–12; 2008.
- [12] Jud , S.; Bedut, S.; Roger, S.; Pinault, M.; Champeroux, P.; White, E.; Le Guennec, J.-Y. Peroxidation of docosahexaenoic acid is responsible for its effects on I TO and I SS in rat ventricular myocytes. *Br. J. Pharmacol.* 139: 816–822; 2003.
- [13] Zhao, Z.; Wen, H.; Fefelova, N.; Allen, C.; Guillaume, N.; Xiao, D.; Huang, C.; Zang, W.; Gwathmey, J. K.; Xie, L.-H. Docosahexaenoic Acid reduces the incidence of early

- afterdepolarizations caused by oxidative stress in rabbit ventricular myocytes. *Front. Physiol.* 3: 252; 2012.
- [14] Roberts, L. J.; Montine, T. J.; Markesbery, W. R.; Tapper, A. R.; Hardy, P.; Chemtob, S.; Dettbarn, W. D.; Morrow, J. D. Formation of isoprostane-like compounds (neuroprostanes) in vivo from docosahexaenoic acid. *J. Biol. Chem.* 273: 13605–13612; 1998.
- [15] Roberts, L. J.; Fessel, J. P.; Davies, S. S. The biochemistry of the isoprostane, neuroprostane, and isofuran Pathways of lipid peroxidation. *Brain Pathol. Zurich Switz.* 15: 143–148; 2005.
- [16] Seet, R. C. S.; Lee, C.-Y. J.; Chan, B. P. L.; Sharma, V. K.; Teoh, H.-L.; Venketasubramanian, N.; Lim, E. C. H.; Chong, W.-L.; Looi, W.-F.; Huang, S.-H.; Ong, B. K. C.; Halliwell, B. Oxidative damage in ischemic stroke revealed using multiple biomarkers. *Stroke J. Cereb. Circ.* 42: 2326–2329; 2011.
- [17] Gladine, C.; Newman, J. W.; Durand, T.; Pedersen, T. L.; Galano, J.-M.; Demougeot, C.; Berdeaux, O.; Pujos-Guillot, E.; Mazur, A.; Comte, B. Lipid profiling following intake of the omega 3 fatty acid DHA identifies the peroxidized metabolites F4-neuroprostanes as the best predictors of atherosclerosis prevention. *PLoS One* 9: e89393; 2014.
- [18] Thireau, J.; Karam, S.; Fauconnier, J.; Roberge, S.; Cassan, C.; Cazorla, O.; Aimond, F.; Lacampagne, A.; Babuty, D.; Richard, S. Functional evidence for an active role of B-type natriuretic peptide in cardiac remodelling and pro-arrhythmogenicity. *Cardiovasc. Res.* 95: 59–68; 2012.
- [19] Podrid, P. J.; Fuchs, T.; Candinas, R. Role of the sympathetic nervous system in the genesis of ventricular arrhythmia. *Circulation* 82: I103–I113; 1990.
- [20] Den Ruijter, H. M.; Berecki, G.; Verkerk, A. O.; Bakker, D.; Baartscheer, A.; Schumacher, C. A.; Belterman, C. N. W.; de Jonge, N.; Fiolet, J. W. T.; Brouwer, I. A.; Coronel, R. Acute administration of fish oil inhibits triggered activity in isolated myocytes from rabbits and patients with heart failure. *Circulation* 117: 536–544; 2008.
- [21] Oger, C.; Brinkmann, Y.; Bouazzaoui, S.; Durand, T.; Galano, J.-M. Stereocontrolled access to isoprostanes via a bicyclo[3.3.0]octene framework. *Org. Lett.* 10: 5087–5090; 2008.
- [22] Oger, C.; Bultel-Poncé, V.; Guy, A.; Balas, L.; Rossi, J.-C.; Durand, T.; Galano, J.-M. The handy use of Brown's P2-Ni catalyst for a skipped diyne deuteration: application to the synthesis of a [D4]-labeled F4t-neuroprostane. *Chem. Weinh. Bergstr. Ger.* 16: 13976–13980; 2010.
- [23] Guy, A.; Oger, C.; Heppekausen, J.; Signorini, C.; De Felice, C.; Fürstner, A.; Durand, T.; Galano, J.-M. Oxygenated metabolites of n-3 polyunsaturated fatty acids as potential oxidative stress biomarkers: total synthesis of 8-F3t-IsoP, 10-F4t-NeuroP and [D4]-10-F4t-NeuroP. *Chem. Weinh. Bergstr. Ger.* 20: 6374–6380; 2014.

- [24] Taber, D. F.; Reddy, P. G.; Arneson, K. O. A potential route to neuroprostanes and isoprostanes: preparation of the four enantiomerically pure diastereomers of 13-F4t-neuroP. *J. Org. Chem.* 73: 3467–3474; 2008.
- [25] Mas, E.; Michel, F.; Guy, A.; Bultel, V.; Falquet, Y.; Chardon, P.; Rossi, J.-C.; Cristol, J. P.; Durand, T. Quantification of urinary F2-isoprostanes with 4(RS)-F4t-neuroprostane as an internal standard using gas chromatography-mass spectrometry Application to polytraumatized patients. *J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci.* 872: 133–140; 2008.
- [26] Berecki, G.; Den Ruijter, H. M.; Verkerk, A. O.; Schumacher, C. A.; Baartscheer, A.; Bakker, D.; Boukens, B. J.; van Ginneken, A. C. G.; Fiolet, J. W. T.; Ophof, T.; Coronel, R. Dietary fish oil reduces the incidence of triggered arrhythmias in pig ventricular myocytes. *Heart Rhythm Off. J. Heart Rhythm Soc.* 4: 1452–1460; 2007.
- [27] Burton, G. W.; Ingold, K. U. Vitamin E as an in vitro and in vivo antioxidant. *Ann. N. Y. Acad. Sci.* 570: 7–22; 1989.
- [28] Janero, D. R.; Hreniuk, D.; Sharif, H. M. Hydrogen peroxide-induced oxidative stress to the mammalian heart-muscle cell (cardiomyocyte): lethal peroxidative membrane injury. *J. Cell. Physiol.* 149: 347–364; 1991.
- [29] Tarnavski, O.; McMullen, J. R.; Schinke, M.; Nie, Q.; Kong, S.; Izumo, S. Mouse cardiac surgery: comprehensive techniques for the generation of mouse models of human diseases and their application for genomic studies. *Physiol. Genomics* 16: 349–360; 2004.
- [30] Hori, M.; Nishida, K. Oxidative stress and left ventricular remodelling after myocardial infarction. *Cardiovasc. Res.* 81: 457–464; 2009.
- [31] Wang, R.; Chai, Q.; Lu, T.; Lee, H.-C. Activation of vascular BK channels by docosahexaenoic acid is dependent on cytochrome P450 epoxygenase activity. *Cardiovasc. Res.* 90: 344–352; 2011.
- [32] De La Torre, A.; Lee, Y. Y.; Oger, C.; Sangild, P. T.; Durand, T.; Lee, J. C.-Y.; Galano, J.-M. Synthesis, discovery, and quantitation of dihom-isofurans: biomarkers for in vivo adrenic acid peroxidation. *Angew. Chem. Int. Ed Engl.* 53: 6249–6252; 2014.
- [33] De la Torre, A.; Lee, Y. Y.; Mazzoni, A.; Guy, A.; Bultel-Poncé, V.; Durand, T.; Oger, C.; Lee, J. C.-Y.; Galano, J.-M. Total syntheses and in vivo quantitation of novel neurofuran and dihom-isofuran derived from docosahexaenoic acid and adrenic acid. *Chemistry.* 21: 2442–2446; 2015.
- [34] Sankaranarayanan, R.; Venetucci, L. Are the anti-arrhythmic effects of omega-3 fatty acids due to modulation of myocardial calcium handling? *Front. Physiol.* 3: 373; 2012.
- [35] Marx, S. O.; Reiken, S.; Hisamatsu, Y.; Jayaraman, T.; Burkhoff, D.; Rosemblyt, N.; Marks, A. R. PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell* 101: 365–376; 2000.

- [36] Fauconnier, J.; Thireau, J.; Reiken, S.; Cassan, C.; Richard, S.; Matecki, S.; Marks, A. R.; Lacampagne, A. Leaky RyR2 trigger ventricular arrhythmias in Duchenne muscular dystrophy. *Proc. Natl. Acad. Sci. U. S. A.* 107: 1559–1564; 2010.
- [37] Fauconnier, J.; Meli, A. C.; Thireau, J.; Roberge, S.; Shan, J.; Sassi, Y.; Reiken, S. R.; Rauzier, J.-M.; Marchand, A.; Chauvier, D.; Cassan, C.; Crozier, C.; Bideaux, P.; Lompré, A.-M.; Jacotot, E.; Marks, A. R.; Lacampagne, A. Ryanodine receptor leak mediated by caspase-8 activation leads to left ventricular injury after myocardial ischemia-reperfusion. *Proc. Natl. Acad. Sci. U. S. A.* 108: 13258–13263; 2011.
- [38] Xie, L.-H.; Weiss, J. N. Arrhythmogenic consequences of intracellular calcium waves. *Am. J. Physiol. Heart Circ. Physiol.* 297: H997–H1002; 2009.
- [39] Cheng, H.; Lederer, W. J.; Cannell, M. B. Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. *Science* 262: 740–744; 1993.
- [40] Negretti, N.; Perez, M. R.; Walker, D.; O'Neill, S. C. Inhibition of sarcoplasmic reticulum function by polyunsaturated fatty acids in intact, isolated myocytes from rat ventricular muscle. *J. Physiol.* 523 Pt 2: 367–375; 2000.
- [41] O'Neill, S. C.; Perez, M. R.; Hammond, K. E.; Shearer, E. A.; Negretti, N. Direct and indirect modulation of rat cardiac sarcoplasmic reticulum function by n-3 polyunsaturated fatty acids. *J. Physiol.* 538: 179–184; 2002.
- [42] Bolli, R. Causative role of oxyradicals in myocardial stunning: a proven hypothesis. A brief review of the evidence demonstrating a major role of reactive oxygen species in several forms of postischemic dysfunction. *Basic Res. Cardiol.* 93: 156–162; 1998.
- [43] Downey, J. M. Free radicals and their involvement during long-term myocardial ischemia and reperfusion. *Annu. Rev. Physiol.* 52: 487–504; 1990.
- [44] Davies, K. J. Protein damage and degradation by oxygen radicals. I. general aspects. *J. Biol. Chem.* 262: 9895–9901; 1987.
- [45] Dizdaroglu, M. Oxidative damage to DNA in mammalian chromatin. *Mutat. Res.* 275: 331–342; 1992.
- [46] Burton, K. P.; Morris, A. C.; Massey, K. D.; Buja, L. M.; Hagler, H. K. Free radicals alter ionic calcium levels and membrane phospholipids in cultured rat ventricular myocytes. *J. Mol. Cell. Cardiol.* 22: 1035–1047; 1990.
- [47] Fukuda, K.; Davies, S. S.; Nakajima, T.; Ong, B.-H.; Kupersmidt, S.; Fessel, J.; Amarnath, V.; Anderson, M. E.; Boyden, P. A.; Viswanathan, P. C.; Roberts, L. J.; Balsler, J. R. Oxidative mediated lipid peroxidation recapitulates proarrhythmic effects on cardiac sodium channels. *Circ. Res.* 97: 1262–1269; 2005.
- [48] Jeong, E.-M.; Liu, M.; Sturdy, M.; Gao, G.; Varghese, S. T.; Sovari, A. A.; Dudley, S. C. Metabolic stress, reactive oxygen species, and arrhythmia. *J. Mol. Cell. Cardiol.* 52: 454–463; 2012.
- [49] Leaf, A.; Xiao, Y. F.; Kang, J. X.; Billman, G. E. Prevention of sudden cardiac death by n-3 polyunsaturated fatty acids. *Pharmacol. Ther.* 98: 355–377; 2003.

- [50] Andersson, D. C.; Marks, A. R. Fixing ryanodine receptor Ca leak - a novel therapeutic strategy for contractile failure in heart and skeletal muscle. *Drug Discov. Today Dis. Mech.* 7: e151–e157; 2010.
- [51] Schrepf, R.; Limmert, T.; Claus Weber, P.; Theisen, K.; Sellmayer, A. Immediate effects of n-3 fatty acid infusion on the induction of sustained ventricular tachycardia. *Lancet* 363: 1441–1442; 2004.
- [52] Marchioli, R.; Barzi, F.; Bomba, E.; Chieffo, C.; Di Gregorio, D.; Di Mascio, R.; Franzosi, M. G.; Geraci, E.; Levantesi, G.; Maggioni, A. P.; Mantini, L.; Marfisi, R. M.; Mastrogiuseppe, G.; Mininni, N.; Nicolosi, G. L.; Santini, M.; Schweiger, C.; Tavazzi, L.; Tognoni, G.; Tucci, C.; Valagussa, F.; GISSI-Prevenzione Investigators. Early protection against sudden death by n-3 polyunsaturated fatty acids after myocardial infarction: time-course analysis of the results of the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione. *Circulation* 105: 1897–1903; 2002.
- [53] De Lorgeril, M.; Salen, P.; Defaye, P.; Rabaeus, M. Recent findings on the health effects of omega-3 fatty acids and statins, and their interactions: do statins inhibit omega-3? *BMC Med.* 11: 5; 2013.
- [54] Von Schacky, C. Omega-3 Index and sudden cardiac death. *Nutrients* 2: 375–388; 2010.
- [55] Lu, P.; Schrag, M. L.; Slaughter, D. E.; Raab, C. E.; Shou, M.; Rodrigues, A. D. Mechanism-based inhibition of human liver microsomal cytochrome P450 1A2 by zileuton, a 5-lipoxygenase inhibitor. *Drug Metab. Dispos. Biol. Fate Chem.* 31: 1352–1360; 2003.
- [56] Peri, K. G.; Almazan, G.; Varma, D. R.; Chemtob, S. A role for protein kinase C alpha in stimulation of prostaglandin G/H synthase-2 transcription by 14,15-epoxyeicosatrienoic acid. *Biochem. Biophys. Res. Commun.* 244: 96–101; 1998.
- [57] Brueggemann, L. I.; Mackie, A. R.; Mani, B. K.; Cribbs, L. L.; Byron, K. L. Differential effects of selective cyclooxygenase-2 inhibitors on vascular smooth muscle ion channels may account for differences in cardiovascular risk profiles. *Mol. Pharmacol.* 76: 1053–1061; 2009.
- [58] Imig, J. D.; Hammock, B. D. Soluble epoxide hydrolase as a therapeutic target for cardiovascular diseases. *Nat. Rev. Drug Discov.* 8: 794–805; 2009.

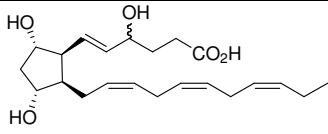
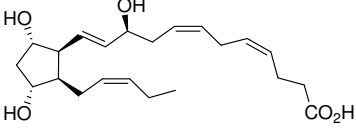
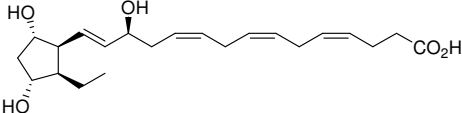
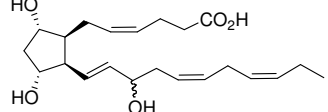
F ₄ -neuroprostanes	Structure	Percentage of reduced arrhythmias
4(<i>RS</i>)-4-F _{4t} -NeuroP		- 86
10-F _{4t} -NeuroP		- 49
13-F _{4t} -NeuroP		- 31
14(<i>RS</i>)-14-F _{4t} -NeuroP		- 43

Table 2. Antiarrhythmic properties of different F₄-neuroprostanes on single cardiac ventricular myocytes. Cardiomyocytes were incubated for 20 minutes in the presence of 1 μM F₄-neuroprostanes prior using the pro-arrhythmic protocol (see Materiel & Methods). Each compound was tested on 30 cells approximately (4-5 mice). Results are expressed in percentage as the reduction of the number of arrhythmic cells after the treatment relative to control (no treatment).

FIGURES

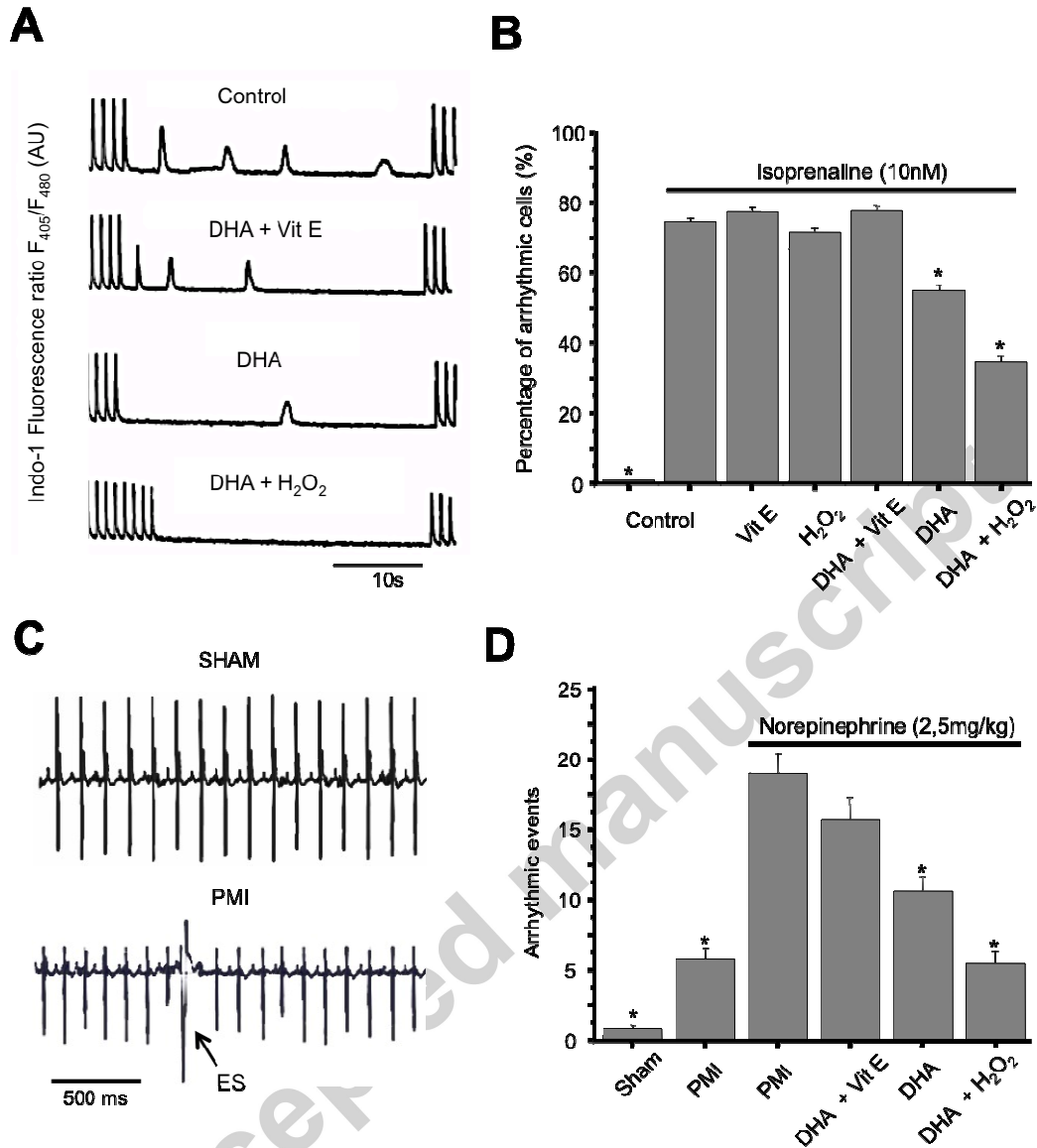


Figure 1. The influence of DHA oxidation on its antiarrhythmic properties. **A)** Representative traces of the effect of 10 μ M DHA in the presence or absence of 1 μ M α -tocopherol (Vit E) or H₂O₂; **B)** Effect of 10 μ M DHA in the presence or absence of 1 μ M α -tocopherol (Vit E) or H₂O₂ on the percentage of arrhythmic cardiomyocytes submitted to 10 nM isoprenaline. α -tocopherol (Vit E) and H₂O₂ were tested alone. Each column bar represents mean \pm SEM, 8-10 cells per isolation (n=8 mice); **C)** Representative traces of ECG recorded in Sham and PMI (post myocardial infarction) mice; **D)** Mean arrhythmic events (mainly extrasystoles, ES) within the hour following norepinephrine (NE) challenge (IP injection in PMI mice, 2.5 mg/kg) in the different treatments indicated. All treatments were prepared in NaCl 0,9% and intravenously injected 20 minutes before the NE challenge (n=11 mice per treatment). * p<0.05 vs control.

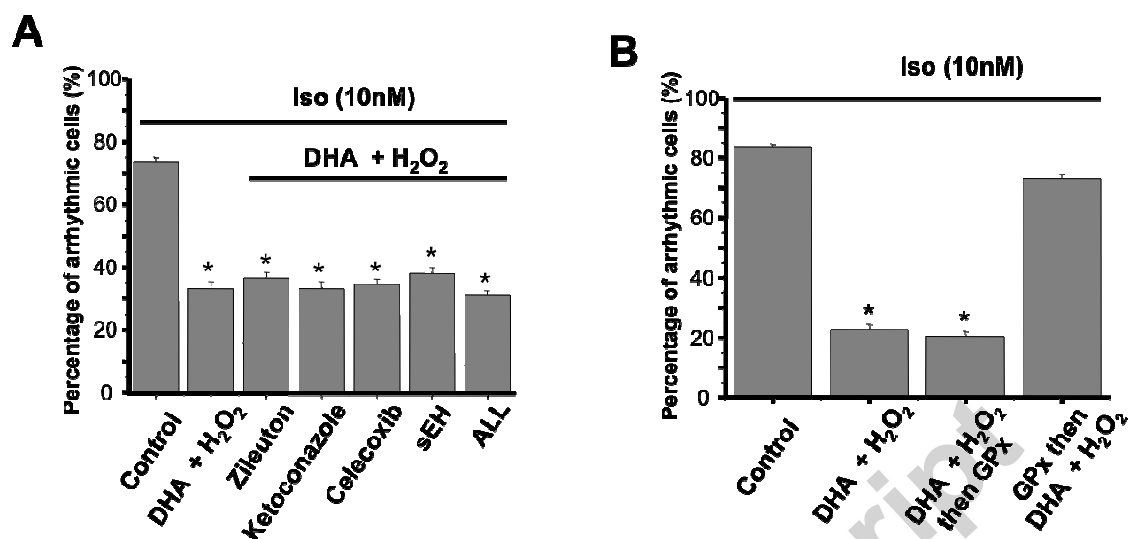


Figure 2. AAP of DHA and oxidized DHA in the presence of enzyme inhibitors. **A)** Effects of different PUFAs enzymatic inhibitors on DHA+H₂O₂ induced reduction of cellular arrhythmias. Zileuton (1 μM), ketoconazole (3 μM) and celecoxib (1 μM) inhibit respectively LOX [55], CYP450 [56] and COX-2 [57]. sEH inhibitor (10 nM) prevents the formation of metabolites of soluble epoxide hydrolase (diols) from EETs [58]. They have been used individually or combined (ALL); **B)** Effects of GPx (10 units, see Methods) on DHA+H₂O₂ induced prevention of cellular arrhythmias. GPx was added before or after DHA+ H₂O₂ were put in the Tyrode solution. * p<0.05 vs control.

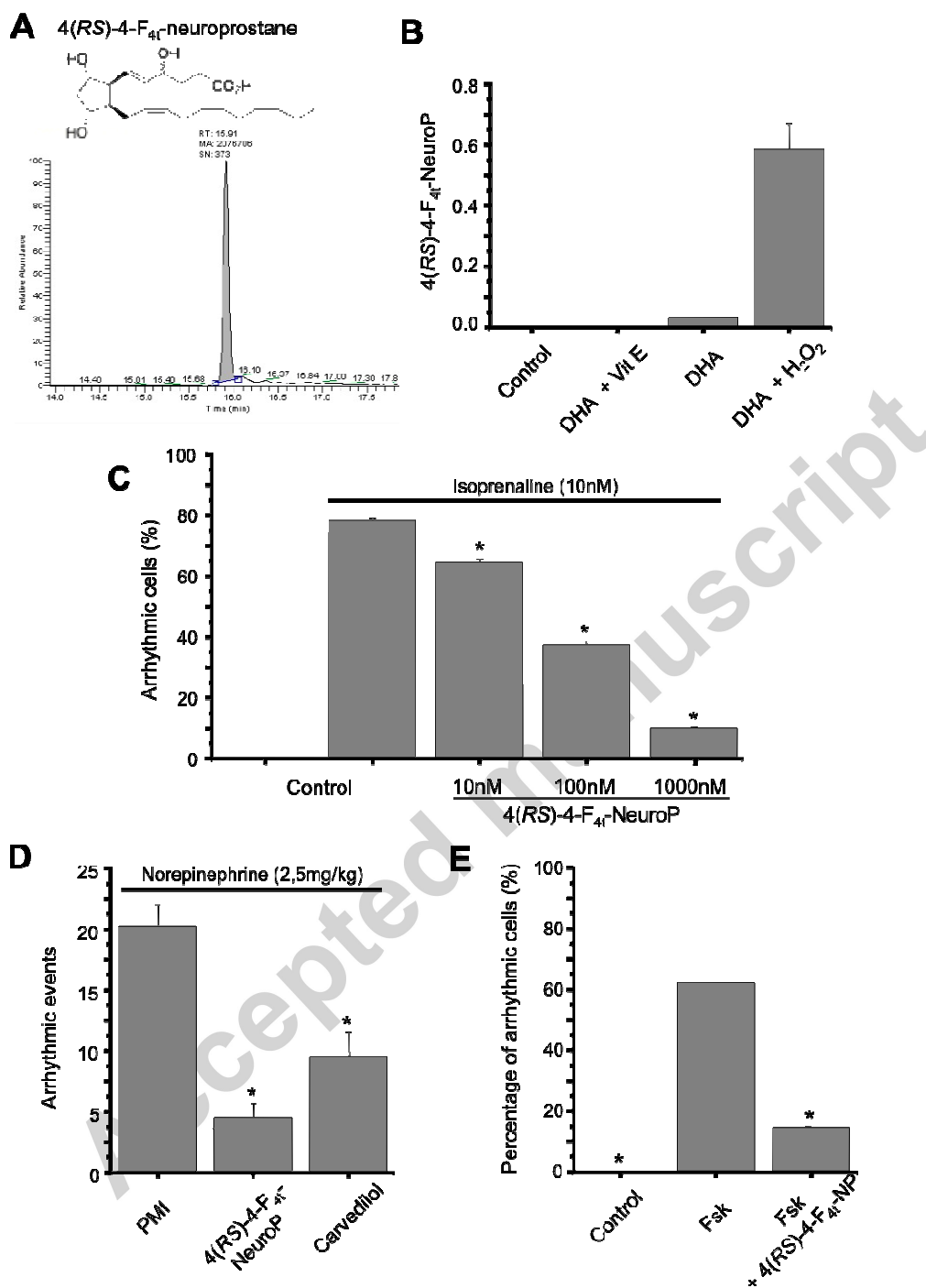


Figure 3. Quantification and the effects of 4(*RS*)-4-F_{4t}-NeuroP on arrhythmias. **A)** Biochemical structure of 4(*RS*)-4-F_{4t}-NeuroP and a typical GC-MS chromatogram profile of 4(*RS*)-4-F_{4t}-NeuroP in sample extract; **B)** Concentration of 4(*RS*)-4-F_{4t}-NeuroP in Tyrode solution incubated with 10 μ M DHA or 10 μ M DHA and 1 μ M α -tocopherol (Vit E) or 10 μ M DHA and 1 μ M H₂O₂; **C)** 4(*RS*)-4-F_{4t}-NeuroP dose-response relationship on the

percentage of arrhythmic cells; **D**) Mean arrhythmic events of extrasystoles within one hour following isoproterenol challenge (IP injection in PMI mice, 2.5 mg/kg) then by an intravenous injection of 4(*RS*)-4-F_{4t}-NeuroP and carvedilol to reach a blood concentration of 1 μM; **E**) Effects of 4(*RS*)-4-F_{4t}-NeuroP on 10 μM forskolin (Fsk) induced cellular arrhythmias. * p<0.05 vs control; ° p<0.05 vs Fsk.

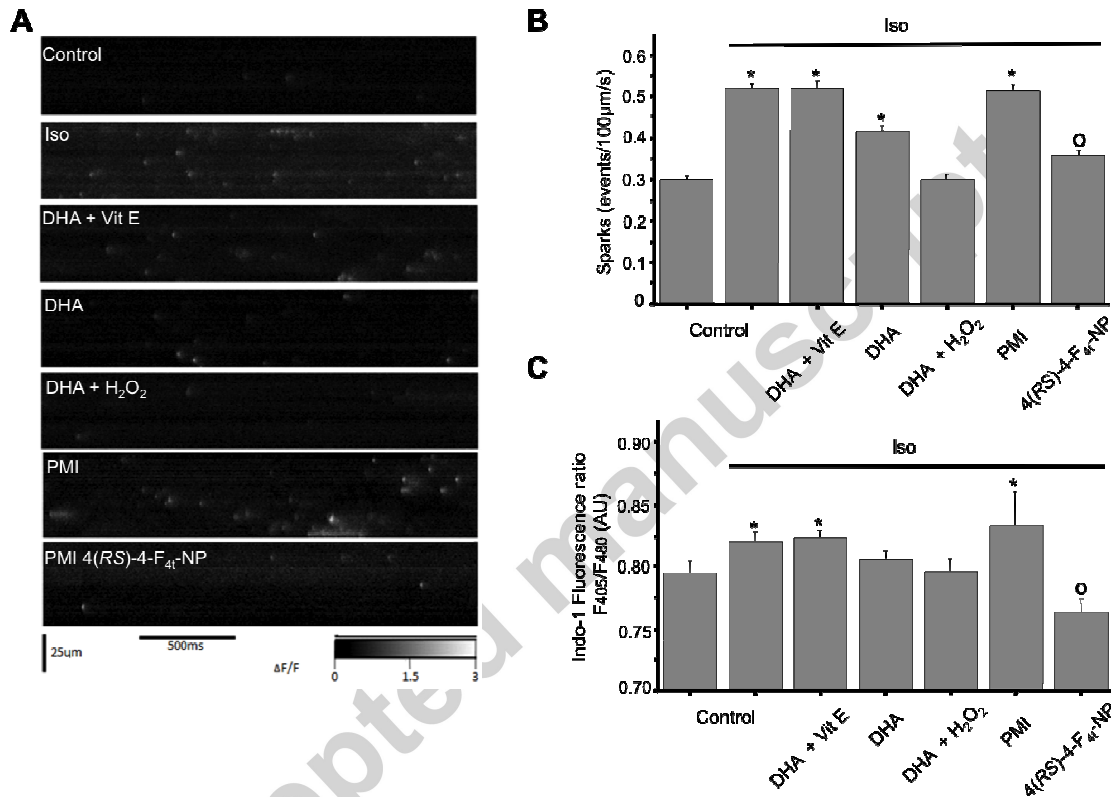


Figure 4. Calcium cycling of the cardiomyocytes. Concentration of treatments indicated are 10 μM DHA, 1 μM α-tocopherol (Vit E), 1 μM H₂O₂ and 1 μM 4(*RS*)-4-F_{4t}-NeuroP. Iso indicates isoprenaline. **A**) Typical line-scan confocal images of spontaneous calcium spark from Fluo-4AM loaded cardiomyocytes in different treatments indicated; **B**) Frequency of calcium sparks measured in **A**. **C**) Effects of the different conditions on diastolic calcium measured in Indo-1AM loaded cardiomyocytes, (expressed as the ratio of fluorescence (F) at 480 and 405 nm). *p<0.05 vs control; °p<0.05 vs PMI.

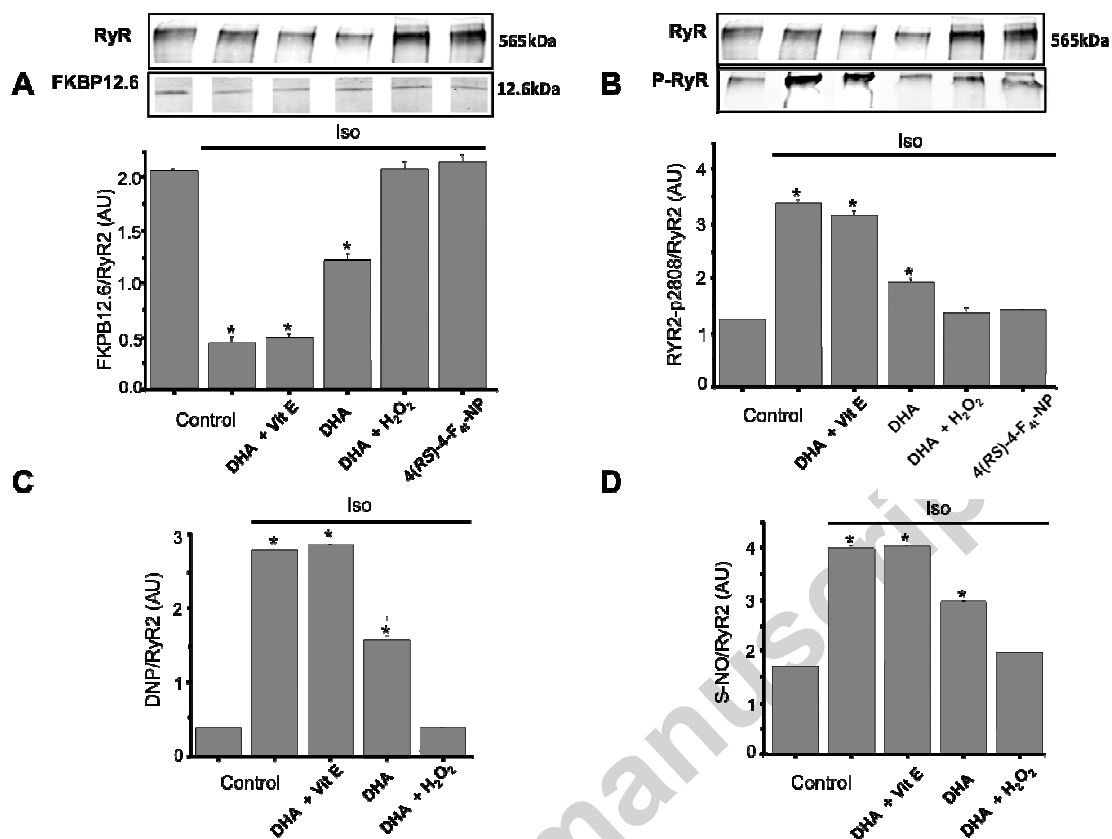
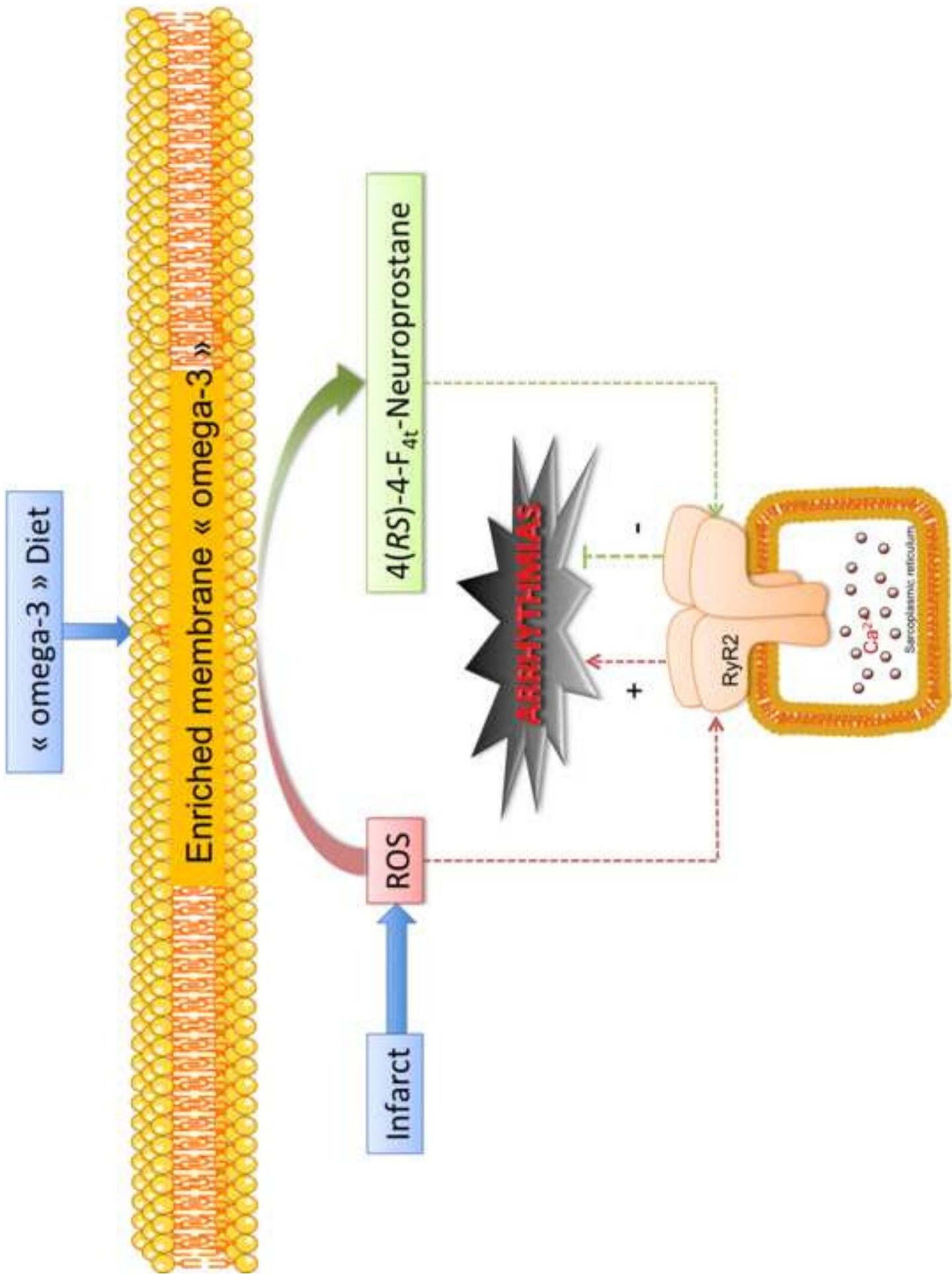


Figure 5. Effects of DHA and derivative on RyR2 posttranslational modifications. Concentration of treatments indicated are 10 μ M DHA, 1 μ M α -tocopherol (Vit E), 1 μ M H₂O₂ and 1 μ M 4(RS)-4-F_{4t}-NeuroP. Iso indicates isoproterenol. Immunoblot expressions of A) FKBP12.6, and B) phosphorylated RyR (RYR2-p2808), C) carbonylated RyR (DNP) and D) S-Nitrosylated RyR (S-NO) against RyR2 are shown after Iso treatment of cardiomyocytes.

Highlights:

- Oxidation of DHA (oxDHA) is a pre-requisite condition for anti-arrhythmic effect.
- 4(*RS*)-4- F_{4t} -neuroprostane from oxDHA displayed potent anti-arrhythmic properties.
- Anti-arrhythmia effect involves stabilization of the FKPB 12.6/RyR2 complex.
- Non-enzymatic metabolites of omega-3 fatty acids have physiological roles.

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TABLE

	SHAM (n = 6)	PMI (n =9)
HR (bpm)	427 ± 29	454 ± 37
EF (%)	60 ± 4	25 ± 12 ***
FS (%)	32 ± 3	12 ± 6 ***
FAC (%)	52 ± 3	19 ± 9 ***
EF (%) B-mode	58 ± 5	12 ± 7 ***
Ao VTI (mm)	49.0 ± 5.8	35.6 ± 4.0 ***

Table 1. Echocardiographic parameters in sham and infarcted mice. HR, heart rate; EF, ejection fraction; FS, fractional shortening; FAC, fractional area change; AoVTI, aortic velocity time integral. Data are represented as mean ± SD. *** p<0.001 for SHAM vs. PMI.