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Oxidative stress
Neuroprostanes
Antiarrhythmic
Cardioprotection
DHA
Ryanodine receptor
Calcium
Free radicals

Neuroprostanes are lipid mediators produced by nonenzymatic free radical peroxidation of docosahexaenoic acid (DHA). DHA is associated with 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 antiarrhythmic properties (AAP) in isolated ventricular cardiomyocytes and in vivo in post-myocardial infarcted mice. Calcium imaging and biochemical experiments indicate that cardiac arrhythmias induced by isoproterenol are associated with Ca^{2+} leak from the sarcoplasmic reticulum after 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 nonenzymatic peroxidation of docosahexaenoic acid can counteract such deleterious effects through cardiac antiarrhythmic properties. Our findings demonstrate 4(*RS*)-4-F_{4t}-NeuroP as a mediator of the cardioprotective AAP of DHA. This discovery opens new perspectives for products of nonenzymatic oxidized ω3 polyunsaturated fatty acids as potent mediators in diseases that involve ryanodine complex destabilization such as ischemic events.

The cardioprotective effects of ω3 polyunsaturated fatty acids (ω3 PUFAs)[2] have been evident since the mid-1970 s [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 and docosahexaenoic acid (DHA), the two major PUFAs of fatty fish, decreased the incidence of SCD in secondary prevention of myocardial infarction [6]. The mechanisms responsible for the antiarrhythmic properties (AAP) of PUFAs remain unclear. In dogs, infusion of a DHA emulsion tended to slow heart rate (HR), shortened the corrected QT interval 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 ω3 PUFAs have direct cardiac electrophysiological effects [11]. However, DHA is highly prone to peroxidation and we have shown that nonenzymatic oxygenated products of DHA and not DHA per se

Abbreviations: 4(*RS*)-4-F_{4t}-NeuroP, 4(*RS*)-4-F_{4t}-neuroprostane; ω3 PUFA, ω3 polyunsaturated fatty acid; AAP, antiarrhythmic properties; COX-2, cyclooxygenase; CYP450, cytochrome P450; DHA, docosahexaenoic acid; DNP, carbonylated RyR; ECG, electrocardiograms; EF, ejection fraction; ES, extrasystoles; F, fluorescence; GPx, glutathione peroxidase enzymes; HR, heart rate; Iso, isoproterenol; LOX, lipoxygenase; LV, left ventricular; NE, norepinephrine; PMI, post-myocardial infarcted; ROS, reactive oxygen specie; RyR2, type 2 ryanodine receptor; SCD, sudden cardiac death; sHE, epoxide hydrolase (diols); SR, sarcoplasmic reticulum; Vit E, vitamin E

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are active on cardiac ionic channels [12]. In agreement, it has been demonstrated in rabbit ventricular cells that early depolarization induced by H_2O_2 is inhibited by DHA, whereas 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 nonenzymatically by ROS initiation and propagation of free radical reactions, leading to the release of numerous products, including neuroprostanes (F_4-NeuroP's) [14]. Neuroprostanes are recognized as oxidative stress biomarkers for the DHA-rich brain and are associated with ischemic stroke and neurodegenerative diseases [15,16]. More recently, it has been proposed that F_4-NeuroP's 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 nonenzymatic oxidation of DHA is a prerequisite for obtaining ventricular antiarrhythmic effects. In particular, one of the F_4-NeuroP isomers, 4(*RS*)-4-F_{4t}-neuroprostane (4(*RS*)-4-F_{4t}-NeuroP), seems to be the main antiarrhythmic metabolite of DHA in preventing deleterious posttranslational modification of RyR2 and thus regulating calcium homeostasis.

Materials and methods

Animal experiments

Male C57Bl/6 mice (Janvier, France) 7 weeks of age 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/O_2, 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 postoperative 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 hygroscopic conditions (23 ± 1 °C, 45 ± 10% humidity, light–dark schedule of 12 h:12 h ad libitum feed).

After 4 weeks, the mice were randomly assigned to the 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 + H_2O_2), and PMI mice treated with 1 μM 4(*RS*)-4-F_{4t}NeuroP. We chose to work on PMI mice challenged with norepinephrine (NE) because it has been shown that the AAP of DHA is secondary to myocardial infarction in human [6].

Treatments were administered as intravenous injections (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 challenged intraperitoneally (ip) with the β1-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, ON, 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 and maintained at 36–38 °C, and electrocardiograms (ECGs) were recorded all along the echocardiographic procedure with limb electrodes. Ejection (EF) and shortening 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 by tracing endocardial end-diastolic and end-systolic areas to estimate left-ventricular volumes, and the endocardial fractional area change on a parasternal short-axis view at papillary muscle level was calculated. Pulsed-wave Doppler of the ascending aortic blood flow was recorded, permitting measurement of the velocity time integral. 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 6F_4NeuroP's. The strategy was based on an easily accessible bicyclic precursor to obtain isoprostanoid derivatives [21], whereas a more refined strategy was 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 (unpublished data). The 13-F_{4t}-NeuroP was synthesized using another strategy [24].

Fatty acid solution and oxidation

To observe antioxidant or oxidant effects, cells were incubated 20 min in Tyrode solution containing 10 μM DHA or 10 μM DHA + 1 μM Vit E or 10 μM DHA + 1 μM H_2O_2. To prepare these solutions, DHA (stock prepared in ethanol) was added to the Tyrode solution after Vit E (stock prepared in chloroform) or H_2O_2 (stock prepared in reverse-osmosis water). The 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 μM DHA was incubated with or without 1 μM α-tocopherol (Vit E) or 1 μM H_2O_2 for 20 min at room temperature. The reaction was terminated with the antioxidant butylated hydroxytoluene (0.005%, w/v). The internal standard C21-15-F_{2t}-IsoP (2.5 ng), synthesized by IBMM (Montpellier, France), was

Table 1
Echocardiographic parameters in sham and infarcted mice.

Parameter	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*
AoVTI (mm)	49.0 ± 5.8	35.6 ± 4.0*

HR, heart rate; EF, ejection fraction; FS, fractional shortening; FAC, fractional area change; AoVTI, aortic velocity time integral. Data are represented as the mean ± SD.

* $p < 0.001$ for sham vs PMI.

added to each sample mix. The sample was further diluted in aqueous sodium acetate solution (pH 4.6), acidified with 1 M HCl, and applied to a prewashed (methanol) Bond Elut Certify II SPE cartridge (Agilent, Santa Clara, CA USA). After the sample was loaded (control, DHA, or DHA + Vit E or DHA + H_2O_2), it was sequentially cleaned with water/methanol (1/1) and hexane/ethyl acetate (7/3) and then the F_4-NeuroP's were eluted with ethyl acetate/methanol (9/1). The eluate was dried under nitrogen and then derivatized at 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 + trimethylsilylchlorosilane 1% and *N,N*-dimethylformamide (2:1) (Sigma–Aldrich, St. Louis, MO, USA). After the reagents were dried under nitrogen, the samples were resuspended in decane.

Gas chromatography–mass spectrometry set at negative ion chemical ionization (NICI; TraceGC and DSQ II mass spectrometer, Thermo Fisher Scientific, Waltham, MA, USA) was used to determine 4(*RS*)-4-F_{4t}-NeuroP [25]. An analytical column (FactorFour; Varian, Palo Alto, CA, USA)-fused silica capillary was used. Helium gas was the carrier gas and the column temperature was programmed from 140 to 250 °C at 30 °C per minute and then 250 to 300 °C at 4 °C per minute and remained at this temperature for 10 min. 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 at *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 to the C21-15-F_{2t}-IsoP internal standard peak.

Preparation of cardiomyocytes

Cellular experiments were performed on freshly isolated left-ventricular myocytes from the noninfarcted free wall (excluding the border zone). In brief, after cervical dislocation, the heart was removed and washed and the aorta was cannulated to a modified Langendorff system. The heart was perfused at a 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 KH_2PO_4, 0.6 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 12 mM $NaHCO_3$, 10 mM $KHCO_3$, 10 mM Hepes (pH 7.4), and 0.1 g/ml liberase dispase (high research grade; Roche, Basel, Switzerland).

After enzymatic treatment (4–6 min), a part of the left ventricle was removed and minced to separate the cells. Isolated myocytes were resuspended in a sterile enzyme-free Tyrode solution, and the Ca^{2+} concentration of the ventricular cell suspension was gradually increased to 1 mM by the addition of $CaCl_2$ in four sequential steps of 100, 100, 300, and 500 μM with a 10-min interval between steps. Finally the cardiomyocytes were kept at room temperature (22–24 °C) until use. Before the treatments, the freshly isolated cardiomyocytes were superfused with standard Tyrode solution (121 mM NaCl, 5.0 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 24 mM $NaHCO_3$, 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 of 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, and anti-epoxide hydrolase (10 nM), from Cayman Chemicals, and a combination of four inhibitors were tested with and without 10 μM DHA + 1 μM H_2O_2 in the cellular arrhythmias. We also used glutathione peroxidase (GPx), which reduces lipid peroxides to alcohols and H_2O_2. A stock solution of GPx was dissolved in water with 10 mM phosphate sodium and 1 mM dithiothreitol. GPx was added either before the DHA + H_2O_2 mix at a concentration of 10 units (1 unit oxide per 1 μM DHA per minute) in Tyrode solution or after the DHA + H_2O_2 mix. For all the experiments, solutions were prepared fresh from the stock and diluted with Tyrode medium.

Calcium channeling

The effects of oxidation of ω3 PUFAs on cell shortening and Ca^{2+} transients of field-stimulated cardiomyocytes were monitored online using a commercial myocyte calcium and contractility monitoring system (IonOptix, Milton, MA, USA) connected to a standard inverted fluorescence microscope. Cells were field-stimulated with 1-ms current pulses delivered via two platinum electrodes. To monitor intracellular Ca^{2+} concentration, cardiomyocytes were loaded with the fluorescent ratiometric Ca^{2+} indicator Indo-1 AM (2 μM, Invitrogen, Grand Island, NY, USA). 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 emits at 405 nm and 480 nm concurrently. The ratio of 405 nm/480 nm indicates the cytosolic Ca^{2+} concentration.

To observe arrhythmias (ventricular extrasystoles), the cells were bathed with 10 nM isoproterenol and stimulated with a 30-s pacing period (1.0 Hz), followed by a 30-s rest period [20,26]. Confocal imaging was performed using a Zeiss LSM510 confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with an argon laser (488 nm) and a 60 ×, 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, Eugene, 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 followed stimulation, myocytes 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 was determined by the intensity of each sequential scan line and plotting the mean intensity as a function of time. The SparkMaster plug-in for ImageJ software was used to detect and analyze Ca^{2+} sparks.

Immunoblot

Proteins were extracted from frozen basal left-ventricular cells (50 mg) homogenized with a manual Polytron instrument. Cells were then lysed in 600 μl extraction buffer (Tris maleate 10 mM, NaF 35 mM, Triton 1%, activated orthovanadate 20 mM, inhibitor cocktail; Roche) for 45 min under rotating agitation. Membrane and cytosolic proteins were collected from the supernatant after 5 min centrifugation at 10,000*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). 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 h at 4 °C. The immune complexes were incubated with protein A/G magnetic beads (Pierce 88802, Rockford, IL, USA) at 4 °C for 2 h, 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 and the DNP-derivatized protein samples formed were detected using an Immunoblot Protein Oxidation Detection Kit (Millipore S7150, Billerica, MA, USA). Proteins were then separated using SDS–PAGE, blotted onto nitrocellulose membranes (0.2 μm; GE Healthcare, Chalfont St Giles, UK), and incubated overnight at 4 °C with primary antibodies: anti-RyR2 (1/1000 dilution; Pierce) and anti-FKBP12.6 (1/1000 dilution; R&D Systems AF 4174, Minneapolis, MN, USA). Protein levels were expressed relative to glyceraldehyde-3-phosphate dehydrogenase (1/60,000 dilution, AB8245; Abcam, Cambridge, MA, USA). All immunoblots were developed and quantified using the Odyssey infrared imaging system (Li-Cor Biosystems, Lincoln, NE, USA) using infrared-labeled anti-mouse and anti-rabbit IgG (1/30,000 dilution) secondary antibodies.

Statistical analysis

All data are given as the 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 Fisher's correction. For paired studies, the Wilcoxon signed rank test was used. Percentage of arrhythmic cells data was analyzed by a χ^2 test. A *p* value of 0.05 or less was taken as statistically significant.

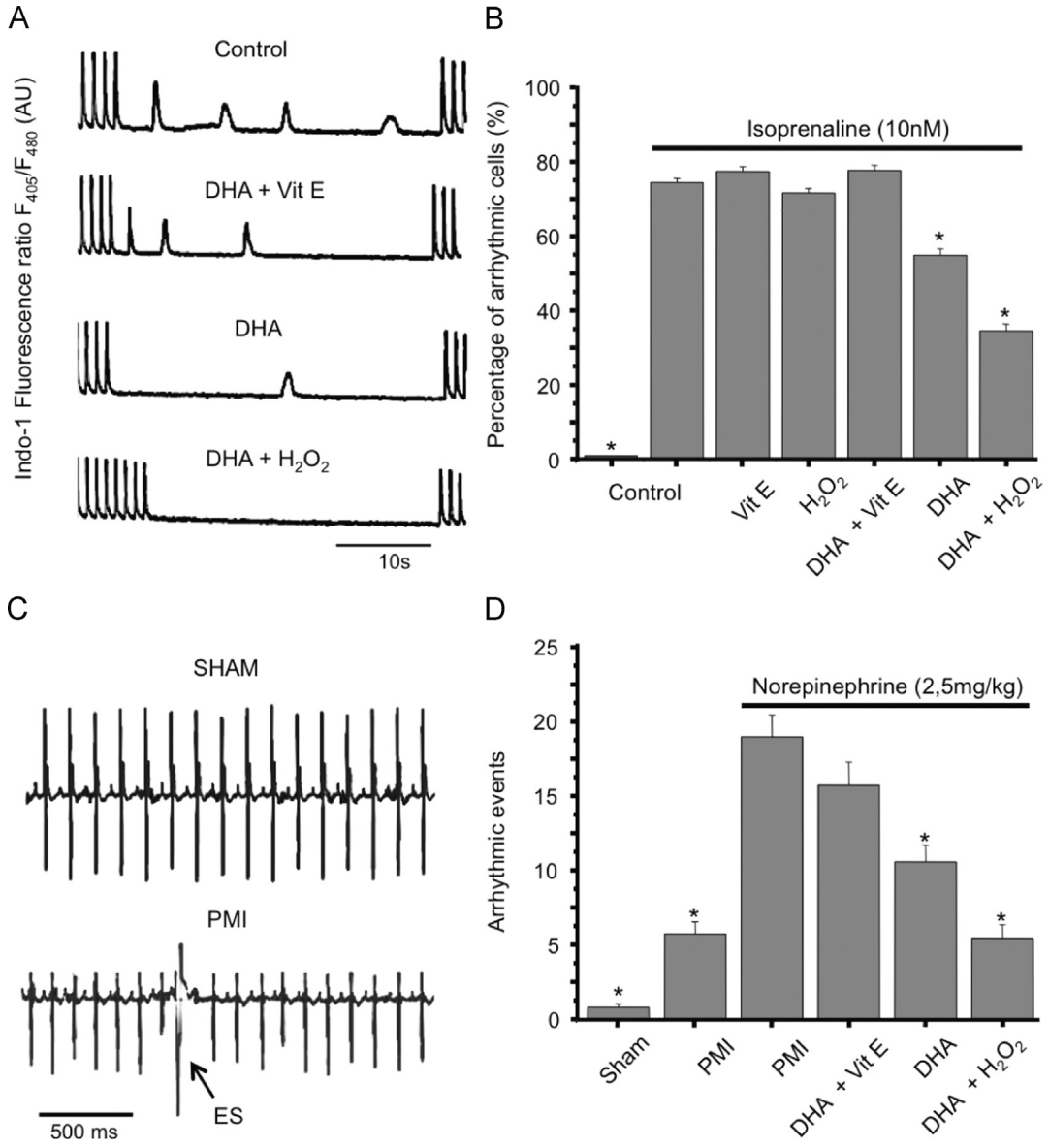


Fig. 1. The influence of DHA oxidation on its antiarrhythmic properties. (A) Representative traces of the effect of 10 μM DHA in the presence or absence of 1 μM α-tocopherol (Vit E) or H_2O_2. (B) Effect of 10 μM DHA in the presence or absence of 1 μM α-tocopherol (Vit E) or H_2O_2 on the percentage of arrhythmic cardiomyocytes subjected to 10 nM isoprenaline. α-Tocopherol (Vit E) and H_2O_2 were tested alone. Each column represents the mean ± SEM, 8–10 cells per isolation ($n = 8$ mice). (C) Representative ECG traces recorded in sham and PMI (post-myocardial infarction) mice. (D) Mean arrhythmic events (mainly extrasystoles) within the hour after norepinephrine (NE) challenge (ip injection in PMI mice, 2.5 mg/kg) in the various treatments indicated. All treatments were prepared in NaCl 0.9% and intravenously injected 20 min before the NE challenge ($n = 11$ mice per treatment). *$p < 0.05$ vs control.

Results

DHA under oxidative stress conditions reduced cardiac arrhythmias

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

Additionally, we investigated the efficacy of DHA at reducing the trigger of ventricular extrasystoles (ESs) in a PMI mouse model established by coronary artery ligation and sensitized by NE [29]. PMI mice develop calcium-dependent ESs due to increased diastolic calcium levels in the 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 into the mice reduced ESs by 45%, which further reinforced the AAP of DHA under pro-oxidant conditions (Fig. 1C and D).

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 were investigated in cardiomyocytes in the presence of various 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 (Fig. 2A). Our observation infers that DHA exerts strong AAP through a nonenzymatic peroxidation process that generates metabolites such as F_4-neuroprostanes and not through a typical enzymatic process that involves, for example, resolvins, protectins, and maresins. To further explore the chemical entities needed for the observed AAP, we buffered any hydroperoxyl derivatives potentially formed by our pro-oxidant conditions (DHA + H_2O_2) by a late addition of GPx (Fig. 2B). AAP were still observed under these conditions, indicating these effects are not related to endoperoxide or hydroperoxide metabolites of DHA. In contrast, arrhythmias persisted when GPx was added before DHA + H_2O_2, indicating that H_2O_2 initiated the formation of the required metabolites of DHA for AAP (Fig. 2B).

Among the F_4-neuroprostanes, 4(RS)-4-F_{4t}-NeuroP had the most active AAP

Supplementation of DHA to atherosclerotic $LDLR^{-/-}$ mice showed that the liver F_4-NeuroP concentration is negatively correlated with atherosclerosis risk [17]. The isomer 4(*RS*)-4-F_{4t}-NeuroP is the most abundant F_4-NeuroP formed from nonenzymatic DHA peroxidation [14]. In our in vitro experiments, incubation of DHA (10 μM) with H_2O_2 (1 μM) in Tyrode solution for 20 min generated 0.61 ± 0.08 μM 4(*RS*)-4-F_{4t}-NeuroP (Fig. 3A and B), whereas DHA with Vit E produced no 4(*RS*)-4-F_{4t}-NeuroP and DHA alone produced trace amounts (0.03 ± 0.01 μM). Furthermore, we recently discovered that levels of 4(*RS*)-4-F_{4t}-NeuroP and another isomer, 10-F_{4t}-neuroprostane (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 antiarrhythmic effect of 4(*RS*)-4-F_{4t}-NeuroP with other F_4-neuroprostanes (10-F_{4t}-NeuroP, 13-F_{4t}-NeuroP, 14(*RS*)-4-F_{4t}-NeuroP; Table 2). Of the four F_4-neuroprostanes tested, 4(*RS*)-4 F_{4t}-NeuroP is the most potent (IC_{50} ≈ 100 nM; Fig. 3C).

Despite the IC_{50} of ≈ 100 nM for 4(*RS*)-4-F_{4t}-NeuroP, we used the maximum concentration (1 μM) to authenticate the AAP in our study. Our in vivo evaluation indicates that the AAP of 1 μM 4(*RS*)-4-F_{4t}-NeuroP were comparable with those of the positive control, 1 μM carvedilol, which is a referenced antiarrhythmic drug (Fig. 3D). Also, 4(*RS*)-4-F_{4t}-NeuroP inhibited arrhythmias produced by the adenylyl cyclase activator forskolin (Fig. 3E), suggesting that β-blocking properties are not involved in the AAP of 4(*RS*)-4-F_{4t}-NeuroP. The absence of bradycardia after 4(*RS*)-4-F_{4t}-NeuroP consolidates this hypothesis (Table 1). Further, the AAP of DHA + H_2O_2 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 + H_2O_2.

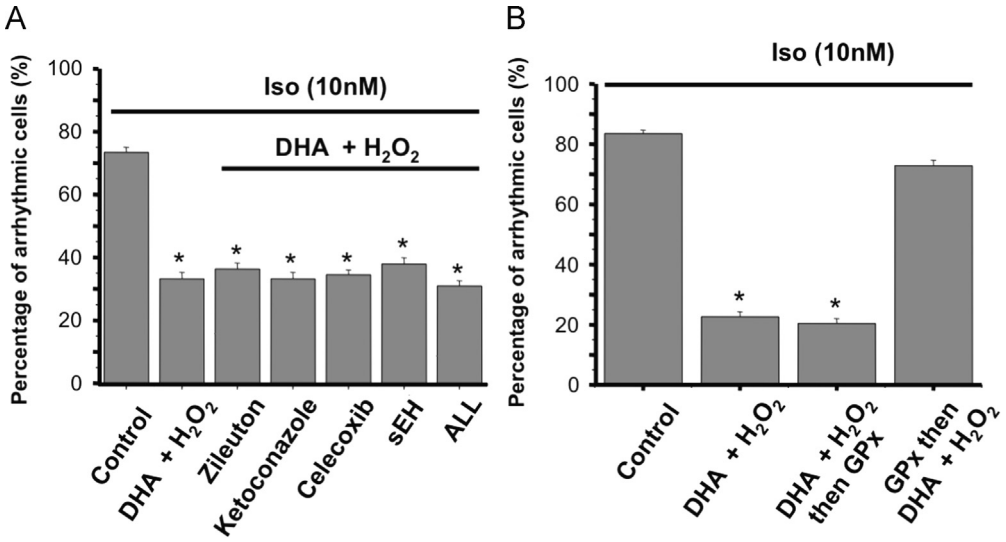


Fig. 2. AAP of DHA and oxidized DHA in the presence of enzyme inhibitors. (A) Effects of various PUFA enzymatic inhibitors on DHA + H_2O_2-induced reduction of cellular arrhythmias. Zileuton (1 μM), ketoconazole (3 μM), and celecoxib (1 μM) inhibit respectively LOX [55], CYP450 [56], and COX-2 [57]. The sEH inhibitor (10 nM) prevents the formation of metabolites of soluble epoxide hydrolase (diols) from EETs [58]. The inhibitors were used individually or combined (ALL). (B) Effects of GPx (10 units, see Materials and methods) on DHA + H_2O_2-induced prevention of cellular arrhythmias. GPx was added before or after DHA + H_2O_2 were put in the Tyrode solution. $^*p < 0.05$ vs control.

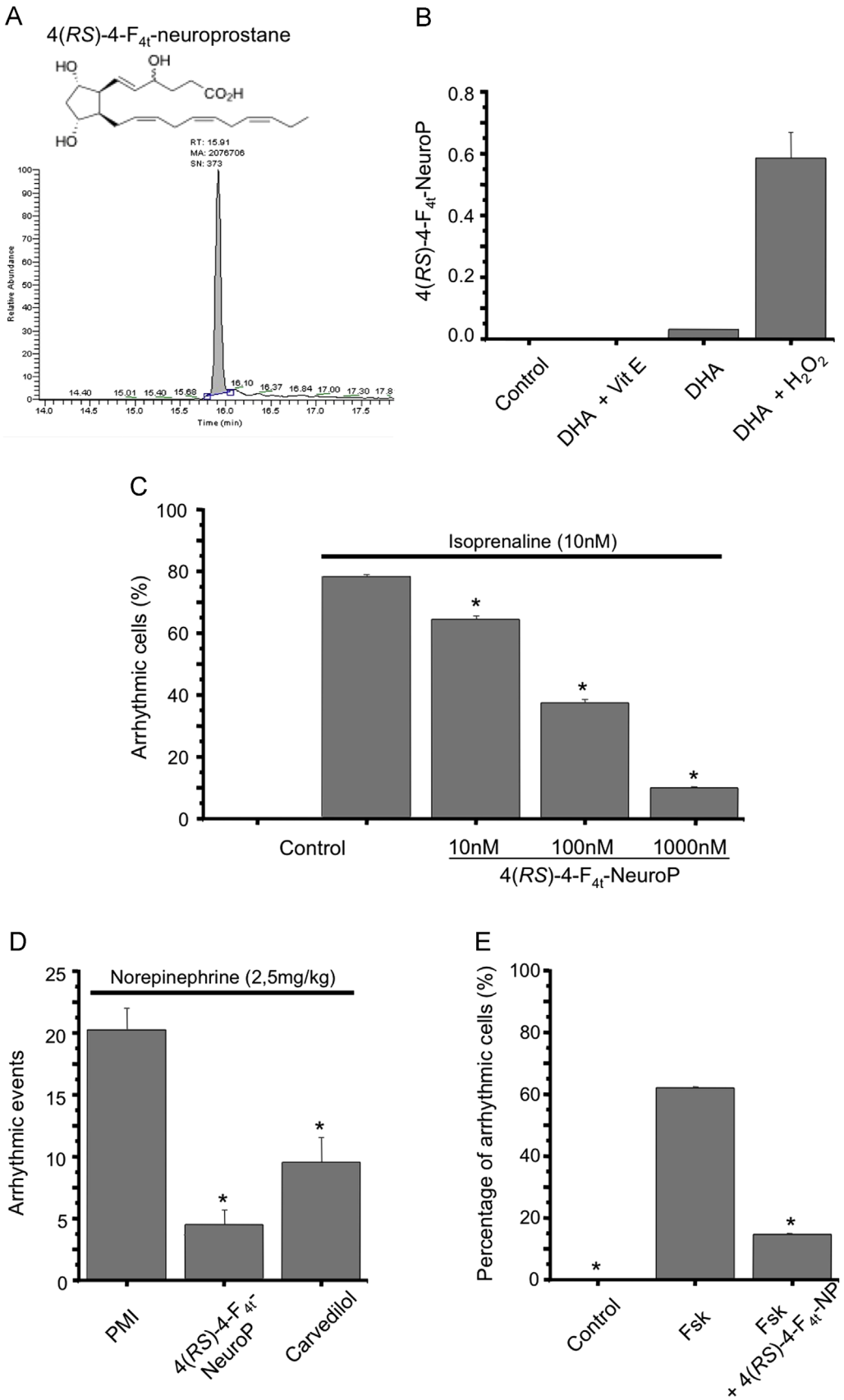
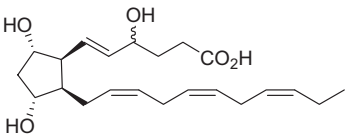
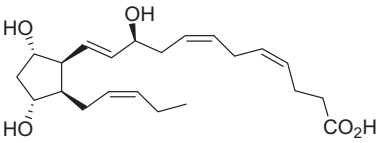
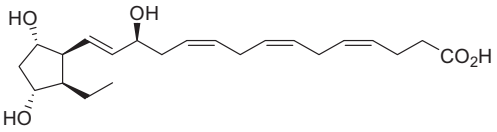
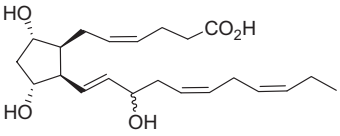


Fig. 3. Quantification and the effects of 4(*RS*)-4-F_{4t}NeuroP on arrhythmias. (A) Biochemical structure of 4(*RS*)-4-F_{4t}NeuroP and a typical GC–MS chromatogram profile of 4 (*RS*)-4-F_{4t}NeuroP in sample extract. (B) Concentration of 4(*RS*)-4-F_{4t}NeuroP in Tyrode solution incubated with 10 μM DHA or 10 μM DHA and 1 μM α-tocopherol (Vit E) or 10 μM DHA and 1 μM H_2O_2. (C) 4(*RS*)-4-F_{4t}NeuroP dose–response relationship expressed as the percentage of arrhythmic cells. (D) Mean arrhythmic events of extrasystole within 1 h of isoproterenol challenge (ip injection in PMI mice, 2.5 mg/kg) and 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 or Fsk.

Table 2
Antiarrhythmic properties of various F_4-neuroprostanes on single cardiac ventricular myocytes.

F_4-neuroprostane	Structure	Percentage of reduced arrhythmias
4(*RS*)-4-F_{4t}-NeuroP		−86
10-F_{4t}-NeuroP		−49
13-F_{4t}-NeuroP		−31
14(*RS*)-14-F_{4t}-NeuroP		−43

Cardiomyocytes were incubated for 20 min in the presence of 1 μM F_4-neuroprostanes before the proarrhythmic protocol (see Materials and methods). Each compound was tested on approximately 30 cells (four or five mice). Results are expressed in percentage as the reduction in the number of arrhythmic cells after the treatment relative to control (no treatment).

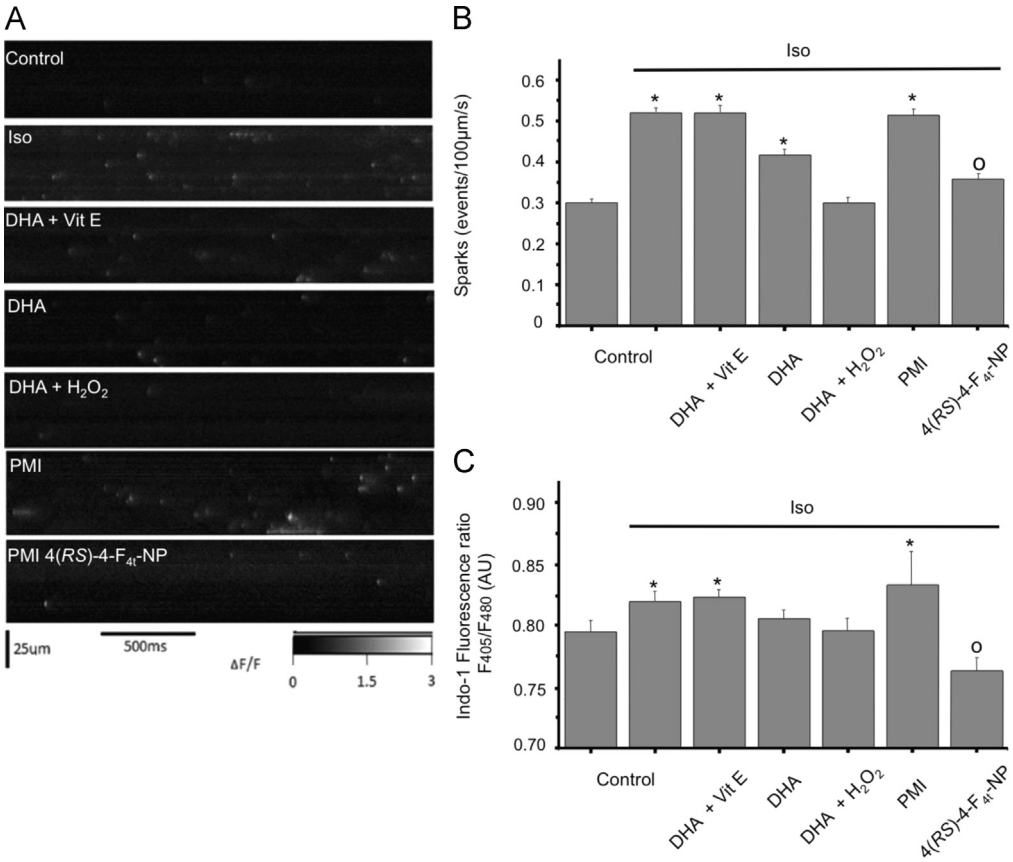


Fig. 4. Calcium cycling of the cardiomyocytes. The concentrations of treatments indicated were 10 μM DHA, 1 μM α-tocopherol (Vit E), 1 μM H_2O_2, and 1 μM 4(*RS*)-4-F_{4t}NeuroP. Iso indicates isoprenaline. (A) Typical line-scan confocal images of spontaneous calcium sparks from Fluo-4-AM-loaded cardiomyocytes under the various treatments indicated. (B) Frequency of calcium sparks measured in (A). (C) Effects of the various conditions on diastolic calcium measured in Indo-1 AM-loaded cardiomyocytes (expressed as the ratio of fluorescence at 480 and 405 nm). $^*p < 0.05$ vs control; $^\circ p < 0.05$ vs PMI.

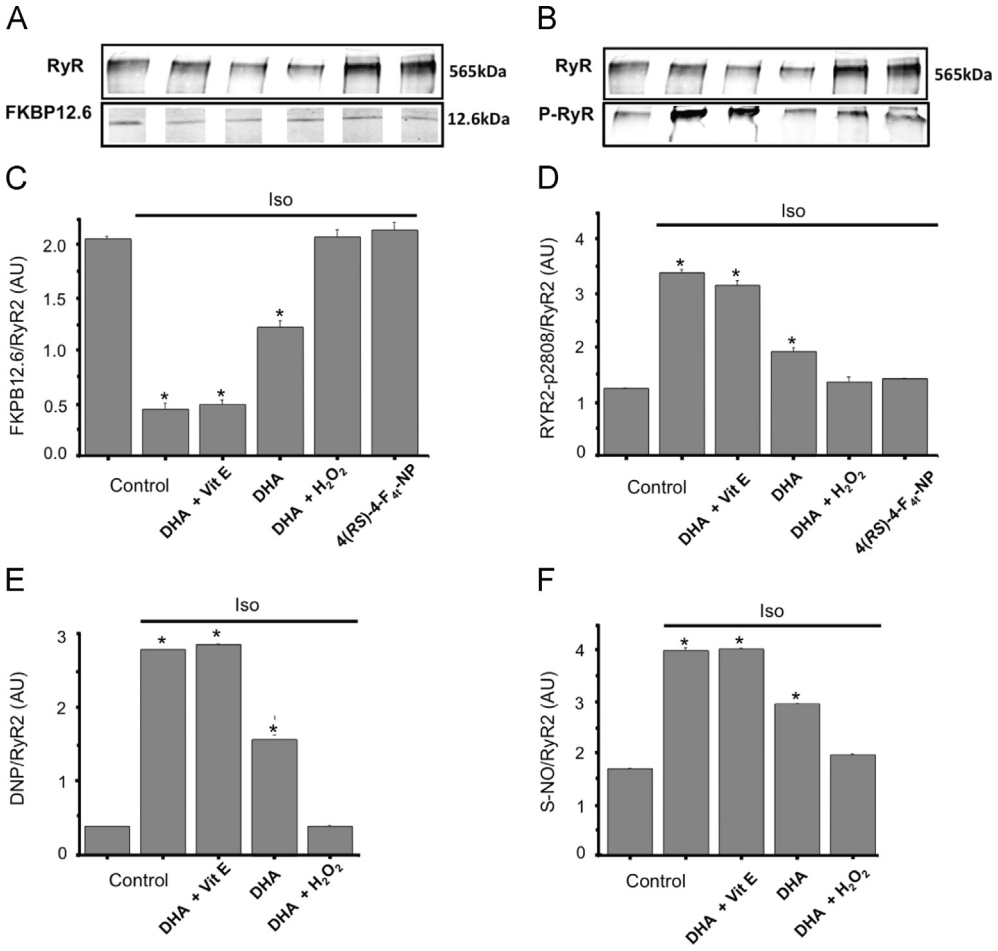


Fig. 5. Effects of DHA and derivative on RyR2 posttranslational modifications. The concentrations of treatments indicated were 10 μM DHA, 1 μM α-tocopherol (Vit E), 1 μM H_2O_2, and 1 μM 4(*RS*)-4-F_{4t}NeuroP. Iso indicates isoproterenol. Immunoblot expression of (A) FKBP12.6 and (B) phosphorylated RyR (RYR2-p2808), (C) carbonylated RyR (DNP), and (D) S-nitrosylated RyR (S-NO) against RyR2 is shown after Iso treatment of cardiomyocytes.

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 ESs. The mechanisms of triggering ESs include alterations in Ca^{2+} homeostasis that are potentiated by catecholamines; the two phenomena are observed after myocardial infarction [34]. The Ca^{2+} signaling alteration could be associated with a leaky RyR2 [35–37]. As a consequence, cytosolic Ca^{2+} levels in diastole rise and promote ESs [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+} spark event frequency was increased by isoprenaline (Fig. 4A and B). This increase was partially prevented by DHA as also shown in previous reports [40,41]. Interestingly, the effects of DHA on Ca^{2+} spark frequency were blunted by α-tocopherol and enhanced by H_2O_2. PMI cardiomyocytes also exhibited an increased frequency of Ca^{2+} sparks that was significantly reduced by 4(*RS*)-4-F_{4t}-NeuroP. The changes in the spark frequency followed changes in resting Ca^{2+} concentration (Fig. 4C).

At the molecular level, posttranslational modifications of RyR2 may account for their leaky behavior [35–37]. We quantified RyR2 carbonylation, S-nitrosylation, and 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 μM DHA, 10 μM DHA + 1 μM Vit E, or 10 μM DHA + 1 μM H_2O_2 (Fig. 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] (Fig. 5A). Iso also promotes RyR2 carbonylation, S-nitrosylation, and phosphorylation on serine 2808 (Fig. 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 (Fig. 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. To verify that the involvement of such an effect is due to a nonenzymatic oxidized lipid product of DHA, we repeated the evaluation on the arrhythmic cardiomyocytes with 4(*RS*)-4-F_{4t}-NeuroP (Fig. 5A and B). The prevention of RyR2 phosphorylation and dissociation of FKBP12.6 of NeuroP was comparable to that of 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 result from the counteraction of RyR2 posttranslational 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 nonenzymatic lipid peroxidation of DHA, particularly 4(*RS*)-4-F_{4t}-NeuroP. This metabolite displayed a unique and unprecedented mode of action among the family of lipid mediators and among 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, in cardiac ischemic diseases and myocardial infarction there is a burst of ROS [42] that can be involved in the generation of arrhythmias, the origin of SCD [43]. Such an abrupt imbalance in 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 postischemic injury that progresses to SCD [47,48]. Classically, there is a consensus that overproduction of ROS is mainly deleterious and does not play a role in normal physiology. For example, it has been shown that an isoketal, E_2-IsoK, originating from the oxidation of arachidonic acid can produce adducts with the sodium channel protein $Na_V1.5$, perturbing its activity in a proarrhythmic way [47]. However, the possibility that nonenzymatic oxygenated metabolites of ω3 PUFAs can exert countereffects has been underinvestigated.

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 contribute to the AAP of DHA [49]. We here demonstrated that the AAP of DHA are dependent on the oxidative status of the environment and that nonenzymatic autoxidation of DHA was a prerequisite of AAP. Our assays pinpointed neuroprostane metabolites as potential metabolites with APP properties. The main isomer, 4(*RS*)-4-F_{4t}-NeuroP, showed potent dose-dependent AAP in cellulo 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 Ca^{2+} leak and normalizing diastolic Ca^{2+} levels and from the prevention of the dissociation of FKBP12.6 from RyR2. Although we cannot 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 posttranslational modifications and RyR2 dysfunction by normalizing RyR2 activity in diastole and thus calcium homeostasis.

Emulsion of ω3 PUFAs injected intravenously has been shown to be antiarrhythmic 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 has to be transformed autoxidatively into neuroprostane for rapid inhibition of arrhythmias.

The prerequisite for DHA oxidation to elicit any AAP could explain the lack of elucidation of the beneficial effects of ω3 PUFAs other than in ischemic cardiovascular diseases [2,52,53]. After initial reports establishing that ω3 PUFAs have cardioprotective properties, the beneficial effects were extended to many if not all cardiovascular problems, even those that are not ischemic [53,54]. This mind-set could be a drawback for the proper development of therapeutic targets in cardiac ischemia and part of the difficulty in justifying its valuable impact in cardioprotection. Overall, the mechanism of action we describe here can explain the favorable impact of ω3 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 under oxidative stress conditions such as ischemic diseases, nonenzymatic 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 nonenzymatically oxygenated metabolites of ω3 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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