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Nonenzymatic lipid mediators, neuroprostanes, exert the antiarrythmic properties of docosahexaenoic acid

Jérôme Roy a, Camille Oger b, Jérôme Threau a, Julien Roussel a, Olivia Mercier-Touzet a, Delinger Faure a,b, Edith Pinot b, Charlotte Farah c, Douglass F. Taber c, Jean-Paul Cristol a, Jetty C.Y. Lee d, Alain Lacampagne a, Jean-Marie Galano b,1, Thierry Durand b,1, Jean-Yves Le Guennec a,b,1

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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 antiarrythmic 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 Ca2+ 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-Fe4-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-Fe4-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-Fe4-neuroprostane derived from nonenzymatic peroxidation of docosahexaenoic acid can counteract such deleterious effects through cardiac antiarrythmic properties. Our findings demonstrate 4(RS)-4-Fe4-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 antiarrythmic 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 arrhythrias [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-Fe4-NeuroP, 4(RS)-4-Fe4-neuroprostane; ω3 PUFAs, ω3 polyunsaturated fatty acid; AAP, antiarrythmic properties; COX-2, cyclooxygenase; CYP450, cytochrome P450; DHA, docosahexaenoic acid; DNP, carboxylated 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 species; RyR2, type 2 ryanodine receptor; SCD, sudden cardiac death; SHE, epoxide hydrolase (diols); SR, sarcoplasmic reticulum; Vit E, vitamin E

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1 These authors contributed equally to this work.
are active on cardiac ionic channels [12]. In agreement, it has been demonstrated in rabbit ventricular cells that early depolarization induced by \( \text{H}_2\text{O}_2 \) 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\(_4\)-NeuroPs) [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\(_4\)-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 nonenzymic oxidation of DHA is a prerequisite for obtaining ventricular antiarrhythmic effects. In particular, one of the F\(_4\)-NeuroP isomers, 4(RS)-4-F\(_4\)t-neuroprostane (4(RS)-4-F\(_4\)t-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 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).

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 \( \mu \text{M} \) DHA (PMI DHA), PMI treated with 10 \( \mu \text{M} \) DHA and 1 \( \mu \text{M} \) \( \alpha \)-tocopherol (PMI DHA + Vit E), PMI mice treated with 10 \( \mu \text{M} \) DHA and 1 \( \mu \text{M} \) hydrogen peroxide (PMI DHA + \( \text{H}_2\text{O}_2 \)), and PMI mice treated with 1 \( \mu \text{M} \) 4(RS)-4-F\(_4\)t-NeuroP. We chose to work on PMI mice challenged with \( \alpha \)-adrenergic agonist norepinephrine (NE) (+H\(_2\text{O}_2\)), and PMI mice treated with 1 \( \mu \text{M} \) DHA (PMI DHA), PMI dosed with vehicle (NaCl 0.9%).

#### Materials and methods

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Treatments were administered as intravenous injections (200 \( \mu \text{l} \)) 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 \( \beta_1 \)-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, 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 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\(_4\)tNeuroP

Using the protocol previously reported, we synthesized 6\( \beta \)-NeuroP. 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\(_4\)t-NeuroP [22], 10-\( \alpha \)-NeuroP [23], and 14 (RS)-14-F\(_4\)t-NeuroP (unpublished data). The 13-\( \alpha \)-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 \( \mu \text{M} \) DHA or 10 \( \mu \text{M} \) DHA + 1 \( \mu \text{M} \) Vit E or 10 \( \mu \text{M} \) DHA + 1 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \). To prepare these solutions, DHA (stock prepared in ethanol) was added to the Tyrode solution after Vit E (stock prepared in chloroform) or \( \text{H}_2\text{O}_2 \) (stock prepared in reverse-osmosis water). The stock solution of 4(RS)-4-F\(_4\)tNeuroP was prepared in Tyrode solution and diluted accordingly for the experimentations.

### Quantification of 4(RS)-4-F\(_4\)tNeuroP

In Tyrode solution (control), 10 \( \mu \text{M} \) DHA was incubated with or without 1 \( \mu \text{M} \) \( \alpha \)-tocopherol (Vit E) or 1 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) for 20 min at room temperature. The reaction was terminated with the antioxidant butylated hydroxytoluene (0.005%, w/v). The internal standard (2.5 ng) was synthesized by IBMM (Montpellier, France), was

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham (n = 6)</th>
<th>PMI (n = 9)</th>
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<tbody>
<tr>
<td>HR (bpm)</td>
<td>427 ± 29</td>
<td>454 ± 37</td>
</tr>
<tr>
<td>EF (%)</td>
<td>60 ± 4</td>
<td>25 ± 12**</td>
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<tr>
<td>FS (%)</td>
<td>32 ± 3</td>
<td>12 ± 6**</td>
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<tr>
<td>FAC (%)</td>
<td>52 ± 3</td>
<td>19 ± 9*</td>
</tr>
<tr>
<td>EF (b-mode)</td>
<td>58 ± 5</td>
<td>12 ± 7**</td>
</tr>
<tr>
<td>AoVTI (mm)</td>
<td>49.0 ± 5.8</td>
<td>35.6 ± 4.0*</td>
</tr>
</tbody>
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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 + H2O2), it was sequentially cleaned with water/methanol (1/1) and hexane/ethyl acetate (7/3) and then the F2-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-diisopropylmethylethylamine 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-F2t-NeuroP [25]. An analytical column (FactorFour; Varian, Palo Alto, CA, USA)-fused silica capillary was used. Helium gas was the carrier gas for a period of 5 min at a flow rate of 30 mL/min followed by a 30-s rest period [20,26].

Calcium channeling

The effects of oxidation of ω3 PUFAs on cell shortening and Ca2+ 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 Ca2+ concentration, cardiomyocytes were loaded with the fluorescent ratiometric Ca2+ 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 Ca2+ 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 Ca2+ concentration.

To observe arrhythmias (ventricular extrasystoles), the cells were bathed with 10 mM 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.

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-cytochrome P450 (2 μM celecoxib), from Sigma–Aldrich, and anti-εepoxide hydrolase (10 nM), from Cayman Chemicals, and a combination of four inhibitors were tested with and without 10 μM DHA + 1 μM H2O2 in the cellular arrhythmias. We also used glutathione peroxidase (GPx), which reduces lipid peroxides to alcohols and H2O2. 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 + H2O2 mix at a concentration of 10 units (1 unit oxidase per 1 μM DHA per minute) in Tyrode solution or after the DHA + H2O2 mix. For all the experiments, solutions were prepared fresh from the stock and diluted with Tyrode medium.

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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 10,000g 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 χ² 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 H2O2. (B) Effect of 10 μM DHA in the presence or absence of 1 μM α-tocopherol (Vit E) or H2O2, on the percentage of arrhythmic cardiomyocytes subjected to 10 nM isoprenaline. α-Tocopherol (Vit E) and H2O2 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 H2O2 [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, H2O2 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 antiarrhythmic effect of 4(RS)-4-F4t-NeuroP for 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 oxidized DHA (Fig. 2A). When applied alone, H2O2 and α-tocopherol had no effect (Fig. 1B), which further supports the role of DHA oxidation on AAP.

Among the F4-neuroprostanes, 4(RS)-4-F4t-NeuroP had the most active AAP

Supplementation of DHA to atherosclerotic LDLR−/− mice showed that the liver F4-NeuroP concentration is negatively correlated with atherosclerosis risk [17]. The isomer 4(RS)-4-F4t-NeuroP is the most abundant F4-NeuroP formed from nonenzymatic 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 4(RS)-4-F4t-NeuroP (Fig. 3A and B), whereas DHA with Vit E produced no 4(RS)-4-F4t-NeuroP and DHA alone produced trace amounts (0.03 ± 0.01 μM). Furthermore, we recently discovered that levels of 4(RS)-4-F4t-NeuroP and another isomer, 10-F4t-neuroprostane (10-F4t-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-F4t-NeuroP in the heart indicates a potential bioactive role. We compared the antiarrhythmic effect of 4(RS)-4-F4t-NeuroP with other F4-neuroprostanes (10-F4t-NeuroP, 13-F4t-NeuroP, 14(RS)-4-F4t-NeuroP; Table 2). Of the four F4-neuroprostanes tested, 4(RS)-4-F4t-NeuroP is the most potent (IC50 ≈ 100 nM; Fig. 3C).

Despite the IC50 of ≈ 100 nM for 4(RS)-4-F4t-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-F4t-NeuroP were comparable with those of the positive control, 1 μM carvedilol, which is a referenced antiarrhythmic drug (Fig. 3D). Also, 4(RS)-4-F4t-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-F4t-NeuroP. The absence of bradycardia after 4(RS)-4-F4t-NeuroP consolidates this hypothesis (Table 1). Further, the AAP of DHA + H2O2 and 4(RS)-4-F4t-NeuroP were similar, suggesting that the AAP of DHA is largely due to the generation of 4(RS)-4-F4t-NeuroP in DHA + H2O2.
Fig. 3. Quantification and the effects of 4(RS)-4-F<sub>4</sub>-NeuroP on arrhythmias. (A) Biochemical structure of 4(RS)-4-F<sub>4</sub>-NeuroP and a typical GC–MS chromatogram profile of 4(RS)-4-F<sub>4</sub>-NeuroP in sample extract. (B) Concentration of 4(RS)-4-F<sub>4</sub>-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<sub>2</sub>O<sub>2</sub>. (C) 4(RS)-4-F<sub>4</sub>-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<sub>4</sub>-NeuroP and carvedilol to reach a blood concentration of 1 μM. (E) Effects of 4(RS)-4-F<sub>4</sub>-NeuroP on 10 μM forskolin (Fsk)-induced cellular arrhythmias. *p < 0.05 vs control or Fsk.
Calcium cycling of the cardiomyocytes. The concentrations of treatments indicated were 10 μM DHA, 1 μM α-tocopherol (Vit E), 1 μM H₂O₂, and 1 μM 4(RS)-4-F₄tNeuroP. 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; †p < 0.05 vs PMI.

<table>
<thead>
<tr>
<th>F₄-neuropropane</th>
<th>Structure</th>
<th>Percentage of reduced arrhythmias</th>
</tr>
</thead>
<tbody>
<tr>
<td>4(RS)-4-F₄t-NeuroP</td>
<td>![Structure Image]</td>
<td>86</td>
</tr>
<tr>
<td>10-F₄t-NeuroP</td>
<td>![Structure Image]</td>
<td>49</td>
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<td>13-F₄t-NeuroP</td>
<td>![Structure Image]</td>
<td>31</td>
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<tr>
<td>14(RS)-14-F₄t-NeuroP</td>
<td>![Structure Image]</td>
<td>43</td>
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Table 2
Antiarrhythmic properties of various F₄-neuroprostanes on single cardiac ventricular myocytes.

Cardiomyocytes were incubated for 20 min in the presence of 1 μM F₄-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).
4(RS)-4-F4t-NeuroP prevents RyR2 dysfunction

In our experimental models, the arrhythmias prevented by 4(RS)-4-F4t-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\(_2\)O\(_2\). PMI cardiomyocytes also exhibited an increased frequency of Ca\(^{2+}\) sparks that was significantly reduced by 4(RS)-4-F4t-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 carbylation, 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 α-tocopherol (Vit E), 10 μM H\(_2\)O\(_2\), and 1 μM 4(RS)-4-F4t-NeuroP. Iso indicates isoproterenol. Immunoblot expression of (A) FKBP12.6 and (B) phosphorylated RyR (RYR2-p2808), (C) carbylated RyR (DNP), and (D) S-nitrosylated RyR (S-NO) against RyR2 is shown after Iso treatment of cardiomyocytes.

**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-F4t-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, E2-Isok, originating from the oxidation of arachidonic acid can produce adducts with the sodium channel protein NaV1.5, perturbing its activity in a proarrhythmic way [47]. However, the possibility that nonenzymatically 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-F4t-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 rcyal-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-F4t-NeuroP in correcting SR Ca2+ leak and normalizing diastolic Ca2+ levels and from the prevention of the dissociation of FKBP12.6 from RyR2. Although we cannot exclude a direct effect of 4(RS)-4-F4t-NeuroP on RyR2, in contrast to rycal effects, we also observed that both oxidized DHA and 4(RS)-4-F4t-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-F4t-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-F4t-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-F4t-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.

Acknowledgments

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References


