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# Non-enzymatic oxidized metabolite of DHA, 4(RS)-4-F<sub>4t</sub>-neuroprostane protects the heart against reperfusion injury

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## A B S T R A C T

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mPTP

Acute myocardial infarction leads to an increase in oxidative stress and lipid peroxidation. 4(RS)-4-F<sub>4t</sub>-Neuroprostane (4-F<sub>4t</sub>-NeuroP) is a mediator produced by non-enzymatic free radical peroxidation of the cardioprotective polyunsaturated fatty acid, docosahexaenoic acid (DHA). In this study, we investigated whether intra-cardiac delivery of 4-F<sub>4t</sub>-NeuroP (0.03 mg/kg) prior to occlusion (ischemia) prevents and protects rat myocardium from reperfusion damages.

Using a rat model of ischemic-reperfusion (I/R), we showed that intra-cardiac infusion of 4-F<sub>4t</sub>-NeuroP significantly decreased infarct size following reperfusion (–27%) and also reduced ventricular arrhythmia score considerably during reperfusion (–41%). Most notably, 4-F<sub>4t</sub>-NeuroP decreased ventricular tachycardia and post-reperfusion lengthening of QT interval. The evaluation of the mitochondrial homeostasis indicates a limitation of mitochondrial swelling in response to Ca<sup>2+</sup> by decreasing the mitochondrial permeability transition pore opening and increasing mitochondria membrane potential. On the other hand, mitochondrial respiration measured by oxygraphy, and mitochondrial ROS production measured with MitoSox red<sup>®</sup> were unchanged. We found decreased cytochrome c release and caspase 3 activity, indicating that 4-F<sub>4t</sub>-NeuroP prevented reperfusion damages and reduced apoptosis. In conclusion, 4-F<sub>4t</sub>-NeuroP derived from DHA was able to protect I/R cardiac injuries by regulating the mitochondrial homeostasis.

## 1. Introduction

Studies on the benefits of dietary n-3 polyunsaturated fatty acids (PUFAs) intake, mainly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), began in the mid-1970's [1,2], and thereafter fish oil consumption received noticeable attention as viable alternative to pharmacological intervention [3,4]. Epidemiological data demonstrated an inverse relationship between fatty fish consumption and risk of coronary heart diseases [5]. More recently, dietary supplementation with fish oil or direct acute DHA infusion was shown to attenuate

myocardial infarct size [6–8], cardiac dysfunction [8–13] and lethal ventricular arrhythmias in ischemia-reperfusion (I/R) animal models [14,15]. Although the cardioprotective mechanisms of DHA and EPA remain to be substantiated, one of the mechanism commonly noted is the modulation of ionic homeostasis and mitochondrial function [12,16,17].

Due to numerous double bonds in PUFAs structure, they are prone to non-enzymatic peroxidation leading to the formation of stable oxidized metabolites. For this reason, oxidized metabolites are often used as biomarkers of oxidative stress in cardiovascular diseases [18].

*Nomenclature and abbreviations:*  $\Delta\Psi_m$ , mitochondrial membrane potential; 4-F<sub>4t</sub>-NeuroP, 4(RS)-4-F<sub>4t</sub>-neuroprostane; n-3 PUFA, n-3 polyunsaturated fatty acids; Ca<sup>2+</sup>, Calcium; CsA, cyclosporine A; CypD, cyclophilin D; DHA, docosahexaenoic acid; ECG, electrocardiogram; EPA, eicosapentaenoic acid; VES, ventricular extrasystoles; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; I/R, ischemia-reperfusion; mPTP, mitochondrial permeability transition pore; TMRM, tetramethylrhodamine methyl ester; ROS, reactive oxygen species; RyR2, type 2 ryanodine receptor; SR, sarcoplasmic reticulum

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Especially, 4(RS)-4-F<sub>4t</sub>-Neuroprostane (4-F<sub>4t</sub>-NeuroP) is one of the main molecule generated from DHA upon non-enzymatic oxidative stress and considered a biomarker for neuro- pathological condition such as brain trauma [19,20] and stroke [21].

Intravenous DHA infusion has been shown to decrease reperfusion arrhythmias and infarct size [6–8,10]. Align to this, 4-F<sub>4t</sub>-NeuroP showed acute anti-arrhythmic properties in a post-myocardial infarction model through the normalization of Ca<sup>2+</sup> homeostasis [22]. However, whether direct administration of 4-F<sub>4t</sub>-NeuroP can confer protection against myocardial ischemia is currently unclear.

In this study, we aimed to determine whether 4-F<sub>4t</sub>-NeuroP exerts cardioprotective effects in a rat model induced with acute myocardial infarction by intracardiac injection of 4-F<sub>4t</sub>-NeuroP *in vivo*, prior to a transient coronary ligation. Reperfusion arrhythmia and infarcted size was subsequently analyzed. Also, 4-F<sub>4t</sub>-NeuroP effects on Ca<sup>2+</sup> homeostasis and mitochondrial function were evaluated at the cellular and subcellular levels.

## 2. Materials and methods

### 2.1. Animal experiments

Five-week-old male Wistar Kyoto rats weighing 220–240 g (Janvier, Le Genest-Saint-Isle, France) were randomly assigned into two groups 1) rats (n=7) received intracardiac infusion of vehicle (0.9% sterile saline solution) and 2) rats (n=7) received final intracardiac infusion of 1 μM (0,03 mg/kg body weight) of 4-F<sub>4t</sub>-NeuroP (Supplementary Fig. 1). The dose given is in accordance to the dose-effect studies published recently [22]. The investigators were blinded to the treatment when measurements were performed. The animal protocol was conducted according to the procedures conformed to European Parliament Directive 2010/63/EU and council on the protection of animals, that was approved by our institutional animal research committee (CEEA-LR-12096). The rats were housed in single cages in a room under 23 ± 1 °C, 45 ± 10% humidity, light-dark schedule of 12 h:12 h and *ad libitum* feed conditions.

For the surgical procedure, the rats were anesthetized by pharmacological injection of ketamine (90 mg/kg) and xylazine (6 mg/kg) mix, and ventilated 60 times per min with a volume-cycled respirator, under ECG monitoring. A medial sternotomy was performed to expose the heart and pericardial. The treatments (4-F<sub>4t</sub>-NeuroP or vehicle) were administrated by intra-cardiac injection (200 μl) of the prepared solution equivalent to 10 times the concentration to reach the final concentrations matching *in cellulo* experiments [22]. The control animals received the same volume of the saline solution plus the equivalent amount of the substrate vehicle (0.9% sterile saline solution). After 20 min from the treatments, the left coronary artery was ligated at 1–2 mm from its origin (5-0 silk suture; Tyco Healthcare, UK) to induce ischemia. In addition, after 45 min of occlusion, the ligation was removed and the left coronary artery was reperfused. Thereafter, the animals had suture with points separated from the various muscular coats to close the surgical zone and for subcutaneous administration of 0.01 mL buprenorphine solution (0.3 mg/mL) as post-operative analgesia.

### 2.2. Measurements of infarct sizes and risk areas

At the end of reperfusion (24 h), the left coronary artery was re-occluded and patent blue dye (5%) was injected in the left atrial appendage to verify the ischemic area *i.e.* area at risk as previously described [23]. Subsequently, all animals were sacrificed by 3 M KCl injection, and the heart tissues were excised into 5–6 cross-sectional slices of 1 mm thickness to quantify the infarction sizes. The slices were then incubated with 1% triphenyltetrazolium chloride in phosphate buffer (pH 7.4) at 37 °C for 20 min. Triphenyltetrazolium chloride forms a red formazan derivative when the tissue is viable and pale

white if necrotic. The morphology of infarct size and area at risk of the left ventricle were quantified by using ImageJ® software 1.46r (National Institutes of Health, USA).

### 2.3. Electrocardiograms acquisition and analyses

The rats were monitored with ECG recordings by a signal transmitter-receiver (RPC-1; DSI) connected to a data acquisition system (Data Sciences International, MN USA). Arrhythmias (ventricular extrasystoles and ventricular tachycardia) and ECG parameters (RR, PR, QRS, and QT interval) were collected continuously over 24 h at a sampling rate of 1000 Hz. The ECG signals were digitally filtered between 0.1 and 1000 Hz and analyzed manually to detect arrhythmias before surgery, during ischemia and over 3 h of reperfusion in animal treated with either the vehicle (0.9% saline solution, *i.c.* injected) or 4-F<sub>4t</sub>-NeuroP. The ECG acquisition and analyses follows the Lambeth conventions for the housing of animal to determine arrhythmic events and were analyzed with Ponemah 5.2 Physiology Platform (USA).

### 2.4. Action potential recording

Action potentials (APs) of papillary muscle or atrial tissue were measured using the microelectrode technique as previously described [24]. APs were triggered by delivering a supra-threshold current at 1 Hz with stimulation electrodes. Glass microelectrodes (GC-150 TF10; Clark Instrument, USA) are pulled using a Narishige puller (PE-2, Japan). The microelectrodes are backfilled with 3 M KCl and connected to an impedance adapter (HS 170, Biologic, France) linked to an amplifier (VF-180, Biologic, France). The microelectrodes have an electrical resistance of about 15–30 MΩ with the reference electrode located in the tissue bath 1–2 cm from the papillary. Only action potentials with amplitude above 80 mV and a resting membrane potential of –70 mV were recorded. Each preparation was equilibrated in the bath for approximately 60 min. When the impalement was stable, the bath was perfused with 4-F<sub>4t</sub>-NeuroP (1 μM) solution for 15–20 min.

The resting membrane potential and action potential durations at 90% of repolarization (APD90) were automatically measured using software LabChart 7.3 Pro (AD Instruments, New Zealand).

### 2.5. Preparation of cardiomyocytes

Cellular experiments were performed from left ventricular myocytes of the non-infarcted wall (excluding the border zone). In brief, the heart tissue was removed, washed and the aorta was cannulated with modified Langendorff system. The heart tissue was then perfused with Ca<sup>2+</sup> free physiological Tyrode solution (116 mM NaCl, 6 mM KCl, 4 mM NaHCO<sub>3</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 1.7 mM MgCl<sub>2</sub>, 21 mM HEPES, 20 mM taurine and 12 mM glucose, pH 7.15) containing a permeant protease inhibitor (E-64, 10 μmol/L, E8640, Sigma-Aldrich, France) at constant flow perfusion rate. After enzymatic treatment for 20 min with type IV collagenase (Worthington, France) solution, a part of the left ventricle was removed and minced to separate the cells. The isolated myocytes were re-suspended in a sterile enzyme-free Tyrode solution, and the Ca<sup>2+</sup> concentration of the ventricular cell suspension was gradually increased to 1 mM by the addition of CaCl<sub>2</sub> in five sequential steps 100, 200, 300 and 500 μM with 10 min intervals in between. Prior to the treatments, the freshly isolated cardiomyocytes were maintained in a physiological solution (140 mM NaCl, 4 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, 1.8 mM CaCl<sub>2</sub> and 11 mM glucose, pH 7.4) at 37 °C for 30 min. Cardiomyocytes with obvious sarcolemmal blebs or spontaneous contractions were omitted from the experiment. Only cardiomyocytes with clear edges were selected and used within 1–6 h after isolation. The cells were incubated with or without 4-F<sub>4t</sub>-NeuroP for 20 min prior to acquisition of each test described below.

## 2.6. Cytosolic calcium and shortening

The effect of 4-F<sub>4t</sub>-NeuroP on cell shortening and Ca<sup>2+</sup> transients of field-stimulated cardiomyocytes was monitored online using commercial myocyte calcium and contractility monitoring system (IonOptix®, Milton, MA, USA) connected to a standard inverted microscope. As explained previously [22], the cardiomyocytes were field-stimulated with 1 ms current pulses delivered *via* two platinum electrodes. To monitor intracellular Ca<sup>2+</sup> concentration, the cardiomyocytes were loaded with the fluorescent ratiometric Ca<sup>2+</sup> indicator Indo-1AM (2 μM, Invitrogen, France), which emit at 405 nm and 480 nm simultaneously. The ratio of 405 nm/480 nm indicates the cytosolic Ca<sup>2+</sup> concentration. Sarcoplasmic reticulum (SR) Ca<sup>2+</sup> content was estimated by massive RyR2 channel opening using caffeine (10 mM).

To observe the activity of RyR2, Ca<sup>2+</sup> sparks observation was determined by confocal imaging (Zeiss LSM510 Carl Zeiss Inc., Oberkochen, Germany) recorded in quiescent myocytes incubated with the Ca<sup>2+</sup> indicator Fluo-4-AM (4 μM) (Molecular Probes, OR USA) [22].

## 2.7. Mitochondrial membrane potential

Tetramethylrhodamine methyl ester (TMRM) was used to measure mitochondrial membrane potential ( $\Delta\Psi_m$ ) [25]. It allows the use of cationic and mitochondrial selective probe to analyze mitochondrial part of the intact cells by confocal microscopy [26] as the TMRM changes the intensity but not the emission spectra in response to  $\Delta\Psi_m$ .

Cardiomyocytes were loaded with TMRM (10 nM) in Tyrode solution for 40 min at 37 °C followed by a washout in Tyrode without TMRM. Confocal images of TMRM fluorescence were obtained by measuring the emitted light at 585 nm after excitation at 568 nm. To minimize the impact of subcellular variability in  $\Delta\Psi_m$ , TMRM fluorescence was measured in five different areas of each cell. The images were taken every 2 min and fluorescence signals were normalized to the fluorescence measured at the start of the experiment, which was set to 100%. In addition, an immunosuppressant inhibitor of mPTP [27] and 100 nM cyclosporine A (CsA) were applied 15 min before data acquisition. At the end of each experiment, the cells were exposed to the mitochondrial uncoupler carbonylcyanure-*p*-trifluoromethoxyphenylhydrazone (20 μM FCCP) to dissipate  $\Delta\Psi_m$  and to determine the dynamics of the dye movements.

## 2.8. Reactive oxygen species (ROS)

The fluorescent MitoSOX Red® dye (5 μM) was used as previously described [28] to measure the dynamic production of superoxide anion within the mitochondria. It has been indicated previously by Zielonka and Kalyanaraman [29], that the change in MitoSox fluorescence measured could not solely be attributed by superoxide production. Therefore, our treatment with or without 4-F<sub>4t</sub>-NeuroP for the MitoSox assessment may not necessary indicate a direct association to superoxide production only. Regardless, it was shown previously, omega-3 PUFA supplementation is associated with an increase in mitochondrial coupling and further augmentation was observed in the presence of H<sub>2</sub>O<sub>2</sub> without affecting the oxidative stress biomarker levels [30]. This suggests that the antioxidant defense, such as superoxide dismutase might be elevated and potentially the balance of oxidant species is affected.

Confocal images were obtained at baseline (first acquisition was done after 1 min of Tyrode perfusion), stimulation at 1 Hz 1 min, 1 Hz 5 min, 3 Hz 1 min and 3 Hz 5 min. The MitoSOX Red® fluorescence was measured in five different areas of each cell. As a positive control, cardiomyocytes paced at 1 Hz were exposed to 1 mM H<sub>2</sub>O<sub>2</sub> at the end of each experiment to increase the mitochondrial [O<sub>2</sub><sup>-</sup>] by inducing product inhibition of the superoxide dismutase in the mitochondrial matrix (SOD2) and thereby inhibit conversion of O<sub>2</sub><sup>-</sup> to H<sub>2</sub>O<sub>2</sub> [31].

## 2.9. Mitochondria permeability

To test the influence of 4-F<sub>4t</sub>-NeuroP on mitochondrial permeability transition pore (mPTP) opening, the mitochondria were isolated from the heart tissues using our previous method [13].

The mPTP opening can be induced by elevated Ca<sup>2+</sup> concentration as well as massive mitochondrial swelling [32], outer membrane rupture and the release of pro-apoptotic factors [17]. In our study, the opening was measured by monitoring the decrease in light scattering associated with mitochondrial swelling at 540 nm using the spectrophotometer. In brief, isolated mitochondria (subsarcolemmal and inter-fibrillar mitochondria, 0.1 mg protein/mL) were re-suspended and diluted in hypo-osmotic buffer B (200 mM saccharose, 5 mM succinate, 10 mM MOPS, 0.01 mM EGTA, 1 mM H<sub>3</sub>PO<sub>4</sub>, pH adjusted at 7.4) and incubated with 4-F<sub>4t</sub>-NeuroP or vehicle, and tested with two concentrations of CaCl<sub>2</sub>, 25 μM and 50 μM to induce mitochondrial swelling by opening the mPTP. The data were collected every min over 1 h interval and were normalized (at 100%) to the data points obtained during the first minute of recording.

## 2.10. Mitochondrial respiration

Respiratory functions of mitochondria were assessed at 37 °C using a Clark-type oxygen electrode, Oxygraph-2K (Oroboros instruments, Innsbruck, Austria). The isolated ventricular myocytes were permeabilized with digitonin and incubated in two sealed thermostat chambers containing 2 mL of respiration medium (0.5 mM EGTA, 3 mM MgCl<sub>2</sub>-6H<sub>2</sub>O, 65 mM KCl, 20 mM Taurine, 10 mM KH<sub>2</sub>PO<sub>4</sub>, 20 mM HEPES, 110 mM sucrose, and 1 g/l BSA, pH 7.1). The following treatments were applied; pyruvate/maleate (5 mM/5 mM, complex I substrates) and succinate (10 mM, complex II substrate) were used as electron donors to specific sites in the electron transport chain, rotenone (10 μM) was added after succinate to inhibit complex I to avoid the reverse electron flow and ATP synthesis was initiated by the addition of 1.5 mM ADP. Thereafter, 1 μM FCCP was added to control the permeabilisation of the ventricular cells. The oxygen consumption was expressed in nmol O<sub>2</sub>.min<sup>-1</sup>.mg protein. Data acquisition and analysis were performed using Oxygraph-2K-DatLab software version 4.3 (Oroboros Instruments, Austria). The fold change was determined as the difference between control and 4-F<sub>4t</sub>-NeuroP groups.

## 2.11. Immunoblot

Protein was extracted (50 mg) from left and right ventricular of I/R rats and then homogenized with a manual polytron® instrument. The tissues were lysed in 1 mL buffer containing Tris maleate 10 mM (pH 6,8), NaF 35 mM, triton 1% and protease inhibitors (Roche 11873580001) for 45 min under rotated agitation and the protein concentration was determined with DC Protein Assay (Biorad, USA). For the detection of caspase 3, 25 μg or 50 μg for the detection of cytochrome c of the total protein were separated on SDS/PAGE gels and transferred onto 0.2 μm nitrocellulose membranes (GE Healthcare, USA) for 1 h at 100 V. The membranes were blocked and then incubated overnight with primary antibodies, anti-cytochrome c (1:1000; ab110325 Abcam, USA), anti-caspase-3 (1:1000; sc7148 Santa Cruz, USA) and GAPDH (1:60000; ab8245 Abcam, USA) at 4 °C. All the immunoblots developed were quantified using the Odyssey infrared imaging system (LI-COR Biosystems, USA) and infrared-labeled secondary antibodies.

## 2.12. Synthesis of 4-F<sub>4t</sub>-NeuroP

Using the protocol previously reported, we synthesized the 4(RS)-4-F<sub>4t</sub>NeuroP. The strategy is based on an easily accessible bicyclic precursor to obtain isoprostanoid derivatives [33].



**Table 1**  
ECG functional parameters measured by implantable telemeters.

		HR	PR interval	QRS interval	QT interval
<b>Control</b>	Vehicle	266.8 ± 15.6	55 ± 2.9	20.8 ± 1.1	140.7 ± 5.3
	4-F <sub>4t</sub> -NeuroP	222.9 ± 16.5*	52.3 ± 2.6	20.6 ± 1.0	111.2 ± 7.0*
<b>Ischemia</b>	Vehicle	299.6 ± 18.2 <sup>+</sup>	62.1 ± 3.8 <sup>+</sup>	25.3 ± 2.3 <sup>+</sup>	159.3 ± 6.9
	4-F <sub>4t</sub> -NeuroP	234.5 ± 19.3*	54.1 ± 1.5*	19.7 ± 0.8*	125.2 ± 5.7*
<b>Reperfusion</b>	Vehicle	279.8 ± 36.2	59.1 ± 3.3	18.8 ± 1.7	147.3 ± 11.7
	4-F <sub>4t</sub> -NeuroP	243.5 ± 26.2	51.6 ± 4.0*	19.7 ± 1.7	111.8 ± 8.3

Mean characteristics of ECG complexes in the different conditions indicated on the left column. Except HR (heart rate) that is expressed in  $\text{beat}\cdot\text{min}^{-1}$ , all interval parameters are expressed in ms. The results are expressed as mean ± SEM, n=7. Statistical differences were annotated against vehicle in the three different conditions. ns = non-significant; 4-F<sub>4t</sub>-NeuroP against vehicle; Ischemia or reperfusion against control.

\* p < 0.05

<sup>+</sup> p < 0.05.

### 2.13. Statistical analysis

All data are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism® (version 6, USA). One-way ANOVA for multiple comparisons was used, followed by a parametric *t*-test with Fishers correction. For paired studies, Wilcoxon signed rank test was used. Percentage of arrhythmic event data was analyzed by  $\chi^2$ -test. A *p*-value of 0.05 or less was considered as statistically significant.

## 3. Results

### 3.1. 4-F<sub>4t</sub>-NeuroP prevented arrhythmic events

We determined the effects of 4-F<sub>4t</sub>-NeuroP on the ECG using telemetric approach (Table 1). An intra-cardiac 4-F<sub>4t</sub>-NeuroP bolus injection significantly decreased the heart rate and this effect was constant after coronary artery ligation. Consequently, the elevation of heart rate, as well as the lengthening of PR, QRS and QT intervals during myocardial ischemia were prevented by 4-F<sub>4t</sub>-NeuroP (Table 1). Simultaneously, the ligation of coronary artery created ventricular extrasystoles (VES) event and 4-F<sub>4t</sub>-NeuroP decreased the incidence of VES during ischemia and following reperfusion by 26% ( $12.0 \pm 2.1$  vs  $16.4 \pm 2.1$ , *p* > 0.05) (Fig. 1A and B), and reduced VES triggered by reperfusion by 41.5% ( $7.2 \pm 1.5$  vs  $12.2 \pm 0.7$ , *p* < 0.01). Ventricular tachycardias, defined as more than five consecutive ectopic beats (Fig. 1C and D), were observed in control and in 4-F<sub>4t</sub>-NeuroP groups. 4-F<sub>4t</sub>-NeuroP treatment tended to lower the number of ventricular tachycardias during ischemia (35%) and reperfusion (30%) events.

### 3.2. 4-F<sub>4t</sub>-NeuroP reduced myocardial infarcted size

Intra-cardiac injection of 4-F<sub>4t</sub>-NeuroP significantly reduced the infarct size (%) (Fig. 2A and B) by about 27% ( $41.0 \pm 2.4\%$  vs  $55.2 \pm 1.2\%$ , *p* < 0.001). Importantly, the area at risk (% of total ischemia area/total area) was not significantly different in control and in 4-F<sub>4t</sub>-NeuroP-treated animals ( $44.6 \pm 3.1\%$  vs  $43.7 \pm 3.2\%$ , *p* > 0.05 respectively), which confirms a homogeneous surgical approach (data not shown).

### 3.3. Effects of 4-F<sub>4t</sub>-NeuroP on calcium homeostasis parameters

Alteration of calcium homeostasis is one of the leading cause of VES after myocardial infarction [34]. At 1 Hz, the application of 4-F<sub>4t</sub>-NeuroP significantly decreased peak steady-state  $\text{Ca}^{2+}$  transients (Fig. 3A and B) without affecting  $\text{Ca}^{2+}$  transient decays (Fig. 3E) suggesting a decrease in SR  $\text{Ca}^{2+}$  release with unchanged SR  $\text{Ca}^{2+}$  reuptake. Consequently, SR  $\text{Ca}^{2+}$  content estimated by caffeine-induced SR  $\text{Ca}^{2+}$  release (10 mM) increased (Fig. 3D) and diastolic  $\text{Ca}^{2+}$  level decreased (Fig. 3C). In order to estimate whether 4-F<sub>4t</sub>-NeuroP

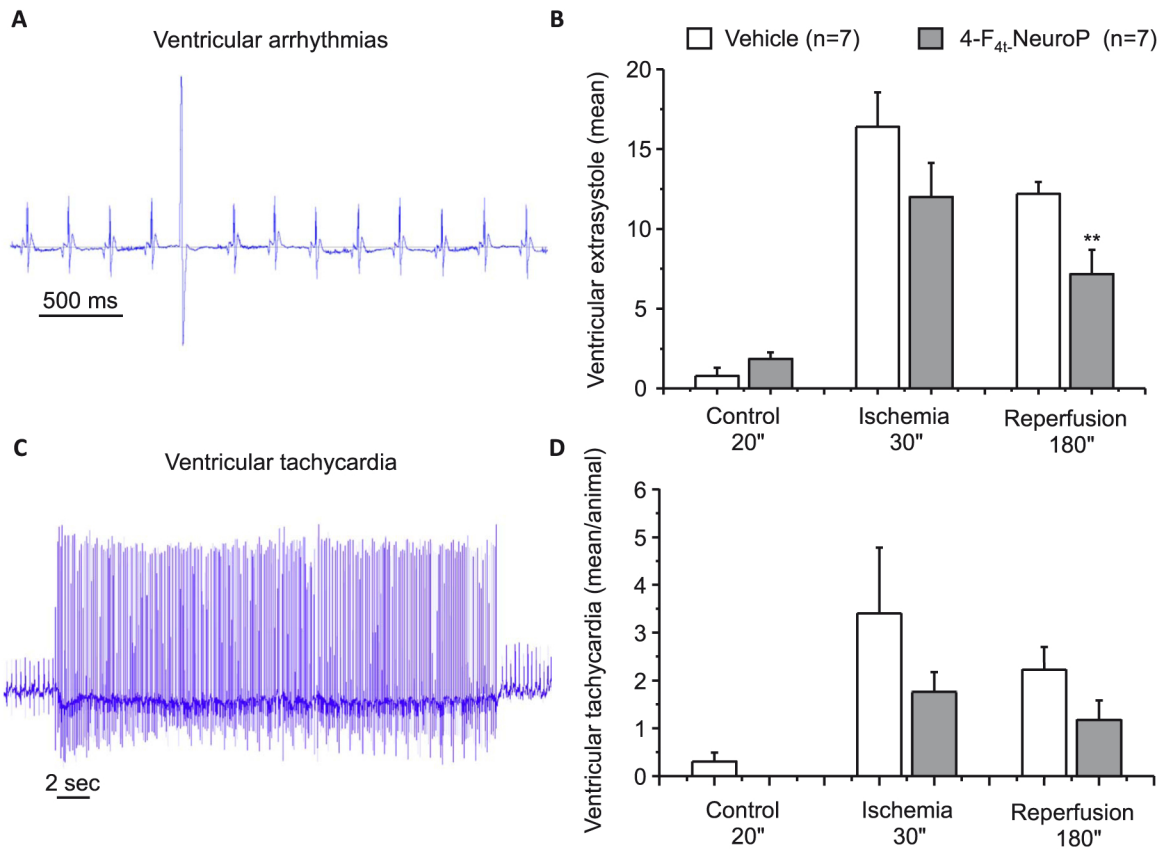
affects RyR2 function, we evaluated the leaky behavior of RyR2 by measuring the frequency of spontaneous  $\text{Ca}^{2+}$  sparks using the confocal microscopy. Cardiomyocytes incubation with 4-F<sub>4t</sub>-NeuroP significantly decreased the spontaneous  $\text{Ca}^{2+}$  sparks frequency (Fig. 3F and G) indicating a decrease in RyR2. SR  $\text{Ca}^{2+}$  transient amplitude depends on both RyR2 open probability and on APD. We thus recorded AP in papillary muscle using microelectrodes (Fig. 3 panels H-J). 4-F<sub>4t</sub>-NeuroP shortened APD at 90% of repolarization (Fig. 3I) and hyperpolarized resting membrane potential in papillary muscle (Fig. 3H). Altogether, our findings suggest that anti-arrhythmic properties of 4-F<sub>4t</sub>-NeuroP may be consecutive to a reduced SR  $\text{Ca}^{2+}$  leak, diastolic membrane hyperpolarization and AP shortening.

### 3.4. Effects of 4-F<sub>4t</sub>-NeuroP on mitochondrial function

A variety of molecular, ionic and energy mechanisms in together determine the electrochemical gradients of the mitochondrial membrane potential ( $\Delta\Psi\text{m}$ ). As the  $\Delta\Psi\text{m}$  is imperative in regulating the activation of apoptotic signaling pathways [35], we determined if 4-F<sub>4t</sub>-NeuroP have an effect on the mitochondrial polarization. Using the tetramethylrhodamine methyl ester (TMRM) fluorescence we tested its accumulation in the mitochondria as a function of the membrane potential.

The application of 4-F<sub>4t</sub>-NeuroP increased the TMRM fluorescence rapidly (Fig. 4A and B) suggesting hyperpolarization of  $\Delta\Psi\text{m}$ . Interestingly, inhibition of cyclophilin D (CypD) with cyclosporine A (CsA) prevented mitochondrial hyperpolarization induced by 4-F<sub>4t</sub>-NeuroP. This hyperpolarization could be explained by a reduced dissipation of proton gradient or an increased oxidative phosphorylation. To test this latter hypothesis, we determined whether 4-F<sub>4t</sub>-NeuroP regulated mitochondrial respiration capacity (Fig. 5B). As shown in Fig. 5B, 4-F<sub>4t</sub>-NeuroP did not influence the maximal mitochondrial respiration capacity suggesting that 4-F<sub>4t</sub>-NeuroP increased coupling efficiency in a cyclophilin D dependent manner.

To determine if 4-F<sub>4t</sub>-NeuroP affects the mPTP, we exposed purified mitochondria to 4-F<sub>4t</sub>-NeuroP or vehicle in the presence or absence of 25  $\mu\text{M}$  and 50  $\mu\text{M}$   $\text{CaCl}_2$ , and monitored the opening of mPTP. Neither 4-F<sub>4t</sub>-NeuroP nor control promoted swelling of the mitochondria throughout 60 min measurement window. In the presence of  $\text{CaCl}_2$ , 4-F<sub>4t</sub>-NeuroP displayed 2.5-fold decrease for both concentrations during the onset of swelling compared to control (Fig. 4C and D) suggesting an inhibition of mPTP opening. Thereafter, we measured the mitochondrial anion superoxide formation under different field stimulation frequency and  $\text{H}_2\text{O}_2$  application. Under these conditions, treatment of 4-F<sub>4t</sub>-NeuroP did not affect the mitochondria anion superoxide production (Fig. 5A).



**Fig. 1.** Effect of 4-F<sub>4t</sub>-NeuroP on ischemia/reperfusion induced arrhythmias. (A) Representative trace of ECG with occurrence of a ventricular extrasystole; (B) Mean number of ventricular extrasystoles in basal condition, during the 30 min of ischemia and after 3 h of reperfusion in the different conditions indicated; (C) Typical spontaneous non-sustained ventricular tachycardia recorded in rats; (D) Mean number of ventricular tachycardia in basal condition, during the 30 min of ischemia and after 3 h of reperfusion in the different conditions indicated. Results are expressed as mean ± SEM, n=7. Statistical differences were annotated against vehicle in the different conditions, \*\*p < 0.01.

### 3.5. Consequences of the mPTP blockade by 4-F<sub>4t</sub>-NeuroP

mPTP opening can cause the release of mitochondrial proteins (caspase 3 and cytochrome c) in the cytosol due to the swelling of mitochondria and rupturing of the outer membrane that subsequently leads to apoptosis [36]. It is known that an I/R episode could induce cytochrome c release from the mitochondria through caspase-dependent pathways [37].

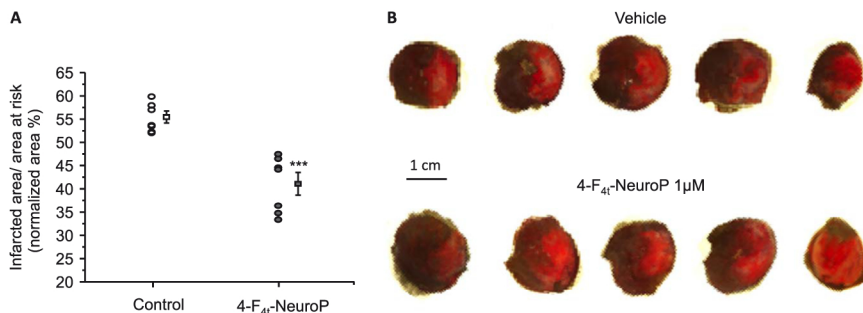
To determine the effects of intra-cardiac perfusion of 4-F<sub>4t</sub>-NeuroP on apoptosis following I/R, we collected the ventricular tissue in the infarcted zone after 24 h of reperfusion. Our measurements revealed that cytochrome c release was decreased in 4-F<sub>4t</sub>-NeuroP groups compared to control in the left ventricular tissue (p < 0.05, Fig. 6A). In parallel, treatment with 4-F<sub>4t</sub>-NeuroP was found to significantly decrease caspase-3 cleavage in the left ventricular tissue by 13% when compared to the control group (p < 0.05, Fig. 6B).

Taken together, these data suggest that the decreased cytochrome c release in the presence of 4-F<sub>4t</sub>-NeuroP coincides with the reduced mPTP opening and apoptotic signaling of the infarcted area.

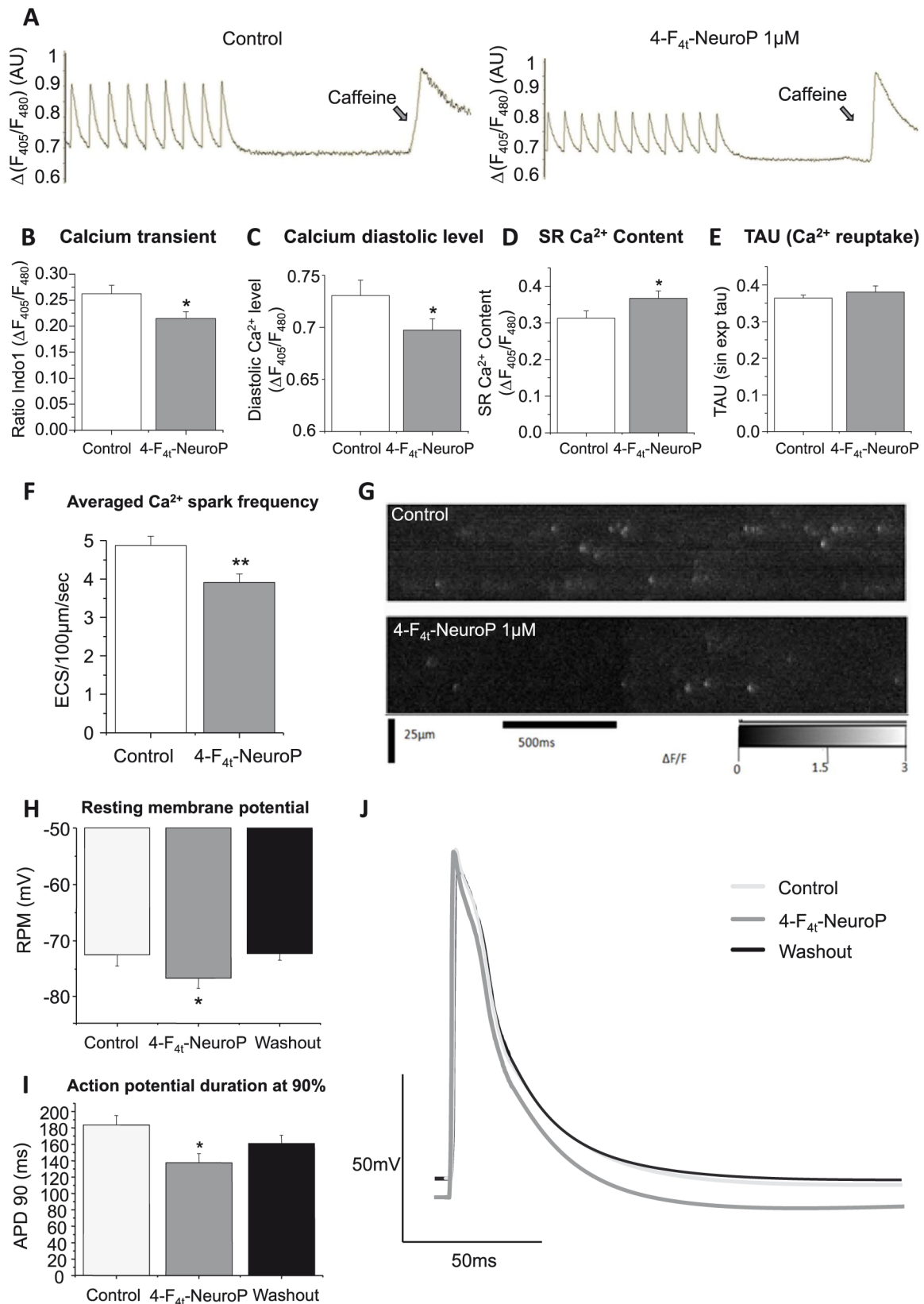
## 4. Discussion

In this study, we provided new insights in understanding the cardioprotective effects of oxygenated metabolites derived from n-3 PUFA. For 30 years, studies have suggested that fish intake or dietary fish oil supplementation, predominantly DHA, reduce atherogenesis and decrease mortality from coronary heart disease, and notably in the prevention of the myocardial infarction and the physiological consequences, that ensue from it [9,38,39].

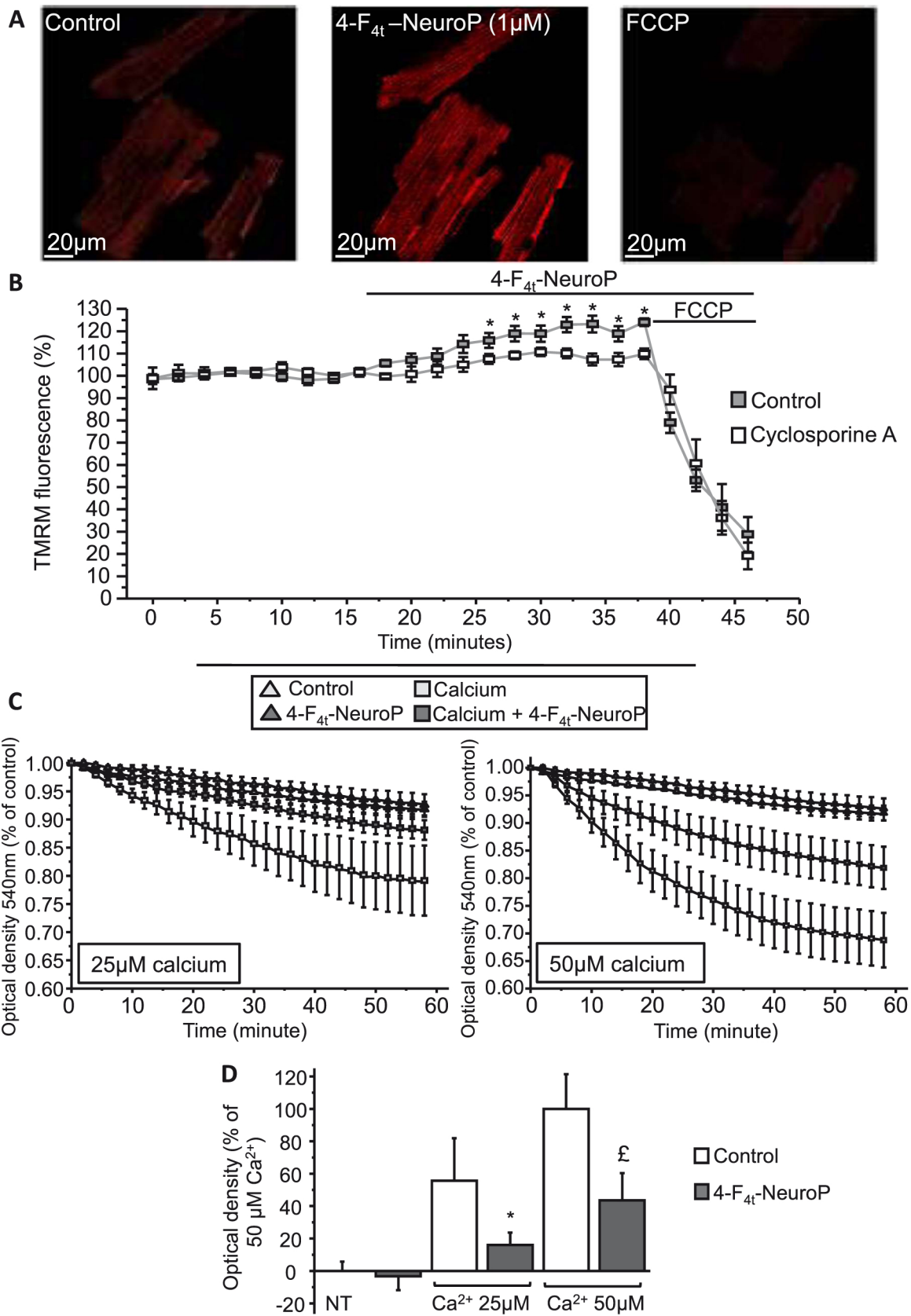
Several investigators have demonstrated that infusion of DHA decreased myocardial infarct size, cardiac dysfunction and frequency of ventricular arrhythmias in animals subjected to coronary artery



**Fig. 2.** *In vivo* effects of 4-F<sub>4t</sub>-NeuroP on ischemia/reperfusion period. The effect of intracardiac infusion of 4-F<sub>4t</sub>-NeuroP on the ratio infarction/area at risk determined (A) by quantification of the area from heart left slices such as those shown in (B). Seven animals were studied in each condition. 4-F<sub>4t</sub>-NeuroP against Control, \* p < 0.05.

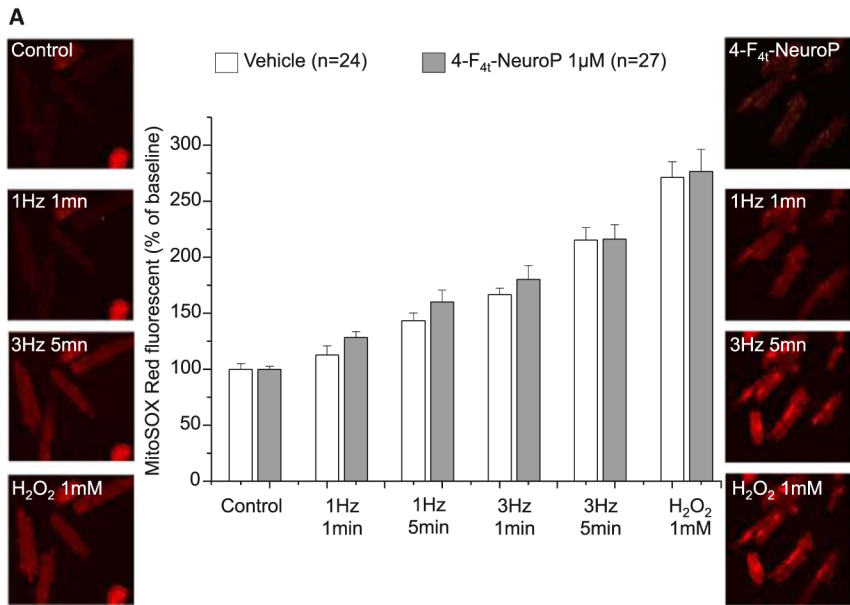


**Fig. 3.** Effect of 4-F<sub>4t</sub>-NeuroP on cellular calcium homeostasis and action potential activity. Typical traces of calcium parameters (A) measured in control and 4-F<sub>4t</sub>-NeuroP conditions in isolated cardiomyocytes on calcium transient amplitude (B), diastolic calcium level (C), averaged SR Ca<sup>2+</sup> content (D) estimated from the caffeine-induced large SR Ca<sup>2+</sup> release (expressed as the ratio of fluorescence at 405 and 480 nm) and Ca<sup>2+</sup> reuptake (E). Mean of frequency of calcium sparks measured in the different conditions indicated (F). Typical line-scan confocal images of spontaneous calcium sparks from Fluo-4-AM loaded cardiomyocytes (G); n=5–6 animals (n=25–30 cells) have been used in each condition. 4-F<sub>4t</sub>-NeuroP vs control, \*p < 0.05. Effects of the 4-F<sub>4t</sub>-NeuroP on resting membrane potential (H), and APD<sub>90</sub> (I). (J) Typical trace of ventricular action potential obtained in different experimental conditions indicated. Mean results from 8 papillaries, control vs 4-F<sub>4t</sub>-NeuroP, \*p < 0.05.

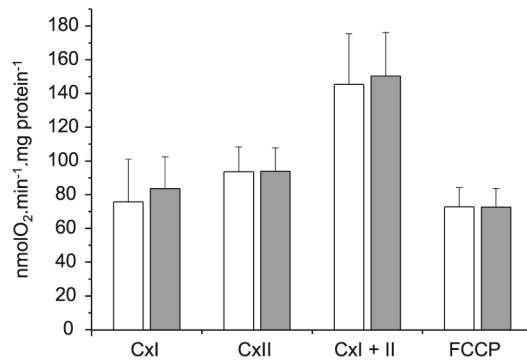


**Fig. 4.** Effect of 4-F<sub>4t</sub>-NeuroP on mitochondrial homeostasis parameters. (A) Typical acquisition of TMRM fluorescence in the different conditions indicated on images; (B) TMRM fluorescent, reflecting  $\Delta\Psi_m$ , before (control and in presence of 4-F<sub>4t</sub>-NeuroP) and after CsA application; (C) Effects of 4-F<sub>4t</sub>-NeuroP on mitochondrial swelling during the application of 25 µM and 50 µM of Ca<sup>2+</sup>; (D) Mean results obtained in different conditions expressed in percentage of swelling 50 µM of Ca<sup>2+</sup>. Five animals (n=25–30 cells) have been used in each condition for TMRM experiments and 4 animals for swelling experiments. Control vs CsA, both following 4-F<sub>4t</sub>-NeuroP application (Panel B), \*p < 0.05; Control vs 4-F<sub>4t</sub>-NeuroP in the presence of 25 µM Ca<sup>2+</sup>, \*p < 0.05; Control vs 4-F<sub>4t</sub>-NeuroP in the presence of 50 µM Ca<sup>2+</sup>, £p < 0.05.





**B** Vehicle (N=7) 4-F<sub>4t</sub>-NeuroP 1µM (N=7)



	Baseline	Complex I	Complex II	Complex I + II	FCCP
Vehicle	9.8 ± 1.5	75.8 ± 25.1	93.6 ± 14.6	145.3 ± 30	72.7 ± 11.4
4-F <sub>4t</sub> -NeuroP	12.2 ± 1.1 ns	83.6 ± 18.9 ns	93.9 ± 13.9 ns	150.4 ± 25.7 ns	72.7 ± 11.1 ns
Fold change	1.4 ± 0.2	1.9 ± 0.7	1.1 ± 0.1	1 ± 0.1	1 ± 0.1

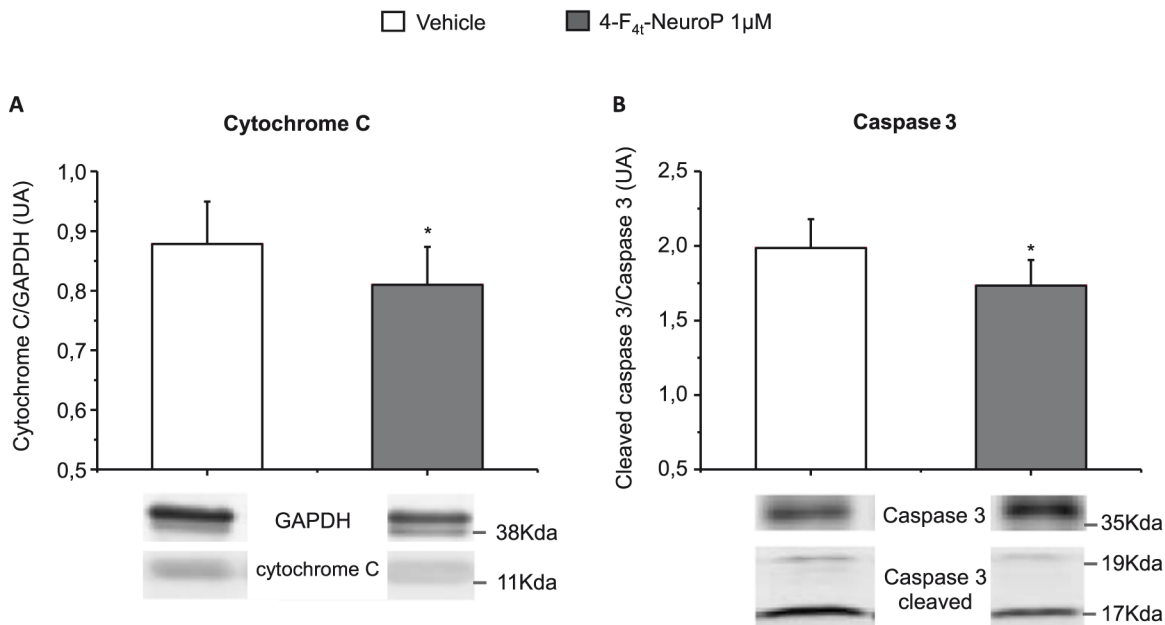
**Fig. 5.** Effect of 4-F<sub>4t</sub>-NeuroP on mitochondrial respiration and ROS production. Typical images and mean ROS production (A) in the different conditions indicated (B). Effects of the 4-F<sub>4t</sub>-NeuroP on different chain respiratory complex expressed in nmolO<sub>2</sub>.min<sup>-1</sup>.mg protein<sup>-1</sup> or arbitrary units for fold change (n=7 animals/experiment). Control vs 4-F<sub>4t</sub>-NeuroP, \*p < 0.05.

occlusion [6–8]. These effects seem to involve the regulation of ionic and mitochondria homeostasis [12,16,17,40]. Also, irrespective to the ratio of EPA:DHA, the cardioprotective effect of dietary fish oils seems to be associated with n-3 PUFA incorporation in the myocardial membrane phospholipids [41,42].

Paradoxically, aside from the well-characterized enzymatic pathways of DHA, its autooxidation from non-enzymatic radical reactions generate a large number of oxygenated metabolites [43]. In particular metabolites that are prostaglandin-like molecules called neuroprostanes (NeuroPs) is noted. After being released by phospholipase A2 from the phospholipid membrane [44], these molecules are metabolized and excreted to body fluids (blood, plasma and urine). They are also known as biomarkers of oxidant injuries [45] and are found elevated in several pathological conditions [46,47] but for several reasons, the bioactive effects of non-enzymatic oxygenated products of n-3 PUFA have been largely ignored and understudied by investigators. We recently showed that 4-F<sub>4t</sub>-NeuroP, possesses anti-arrhythmic properties in a post myocardial infarction model, through the modulation of Ca<sup>2+</sup> home-

ostasis [22]. There are other evidences that peroxidized lipids from PUFA can be bioactive [48], particularly in stress conditions accompanying I/R period in the cells and tissues that are highly plastic and adaptable, such as the heart [49]. Interestingly, these studies clearly demonstrated that some biological properties of the n-3 PUFA depended on their peroxidation but the exact nature of molecules were not determined [11,50–52]. It has been shown in a mice model of atheroma (LDLR<sup>-/-</sup> mice) that fish oil could help to prevent atherosclerosis [53]. Follow-up, it has been shown that 4-F<sub>4t</sub>-NeuroP is inversely related to the size of the atheroma plaque suggesting a beneficial biological role but further elucidation was not made [54].

In the present study we show that the infusion of 4-F<sub>4t</sub>-NeuroP before I/R reduced myocardial injury and dysfunction. Intra-cardiac delivery of 4-F<sub>4t</sub>-NeuroP significantly reduced reperfusion-induced infarct sizes, decreased the occurrence of ventricular arrhythmia during reperfusion and the total number of ventricular tachycardia. One potential mechanism for the cardiac protection is the bradycardia effect of 4-F<sub>4t</sub>-NeuroP, which could lower myocardial oxygen consumption



**Fig. 6.** Effect of 4-F<sub>4t</sub>-NeuroP on pro-apoptotic factors. (A) Measurement of cytochrome c and caspase 3 protein; (B) in ventricular tissue after ischemia/reperfusion period in control condition and after 4-F<sub>4t</sub>-NeuroP infusion (n = 5 animals/experiments). Control vs 4-F<sub>4t</sub>-NeuroP, \*p < 0.05.

during ischemia and decrease mitochondrial dysfunction, and therefore reduce apoptosis pathway. Activation of vagal tone by n-3 PUFA was previously suggested [55,56] and the direct effect on cardiomyocytes by 4-F<sub>4t</sub>-NeuroP was confirmed in this study.

In addition, we demonstrated increased  $\Delta\Psi_m$  without altering maximal mitochondrial function (Fig. 5B) suggesting increased respiration coupling efficiency. Indeed, it was demonstrated in human striated skeletal muscle that n-3 PUFA supplementation, failed to have an impact on maximal mitochondria respiration despite a change in the inner mitochondrial membrane composition. However, mitochondrial respiration sensitivity to ADP was enhanced, suggesting an increase in coupling efficiency, presumably through a change in ATP synthase and/or ANT activity [30]. Interestingly, the CypD and CsA also regulate the ATP synthase and/or the ANT, which have also been shown to be part of mPTP. Notwithstanding, it was also shown that H<sub>2</sub>O<sub>2</sub> production slightly increased the mitochondria originated from n-3 PUFA supplemented muscle whereas the oxidative stress biomarker levels were not altered. Taken together, although the involvement DHA metabolites in the regulation of mitochondrial function after n-3 PUFA supplementation remained to be established and are currently under investigation for further studies, one can speculate that the 4-F<sub>4t</sub>-NeuroP effects on mitochondrial function and mPTP opening may at least in part be related to the modulation of ATP synthase and/or ANT activity.

Apoptosis and necrosis are the major form of cell death after a short period of ischemia followed by reperfusion [57]. It is known that mPTP opening induces mitochondrial swelling and outer membrane disruption. Subsequent cytochrome c releases in the cytosol activate caspase-9 and apoptosome formation, and finally leads to the cleavage of the executioner caspase-3. 4-F<sub>4t</sub>-NeuroP down regulated the markers of mitochondrial stress (mPTP opening and cytochrome c release), indicating that 4-F<sub>4t</sub>-NeuroP could carry out anti-apoptotic action (caspase-3 activity) mainly through the attenuation of mitochondrial dysfunction by modulating mPTP opening.

We showed recently that DHA in an oxidative stress environment reduces the frequency of spontaneous Ca<sup>2+</sup> spark events [22]. This observation is associated to EPA inhibition of open probability (Po) for RyR2 channels incorporated into planar lipid bilayers [58]. RyR2 dysfunction is known as one of the primary pathophysiological mechanism involved during I/R. This is confirmed by the use of pharmacological agents that are able to prevent RyR2 dysfunction from

reperfusion injuries. Therefore, the effects of 4-F<sub>4t</sub>-NeuroP reducing RyR2-dependent SR Ca<sup>2+</sup> leak could explain the electrophysiological perturbations during reperfusion. Indeed, besides protective effects on ventricular arrhythmia, decreased RyR2 activity may also have an impact on pacemaker activity and reduce heart rate [59]. This protective effect is also explained by the hyperpolarization of cardiac cells associated with a decrease in APD (Fig. 3H-J).

The present study also revealed the implication of mitochondria and the benefits of n-3 PUFA. During I/R, it is known that the opening of mPTP may play a causative role in post-reperfusion necrotic death of the cardiac myocytes [60–62]. Following myocardial reperfusion after ischemia, several incidences occur simultaneously namely oxidative stress, mitochondrial Ca<sup>2+</sup> overload and loss of ATP concentration. This leads to mPTP opening and could be disadvantageous [63] as it is reported that inhibition of mPTP opening with pharmacological interventions or genetic modifications limit infarct size [64]. Numerous studies have suggested that dietary n-3 PUFA are protective against Ca<sup>2+</sup>-induced opening of the mPTP [17] and mitochondrial swelling is prevented by n-3 PUFA treatment [40]. Both phenomenon are known to cause outer membrane rupture and release of cytochrome c and other factors from the intermembrane space into the cytosol thereby initiate apoptotic signaling [65].

Our results demonstrate that 4-F<sub>4t</sub>-NeuroP induces  $\Delta\Psi_m$  leading to hyperpolarization. This effect is not due to an augmentation of the mitochondrial respiratory chain activity or of the oxidative phosphorylation [66] but it is due to an inhibition of mPTP. In addition, 4-F<sub>4t</sub>-NeuroP confers protection against Ca<sup>2+</sup>-overload injuries in the mitochondria that is linked to decreased mPTP opening that subsequently prevents the release of pro-apoptotic factor such as the cytochrome c and the activation of the caspase 3.

Beside the comprehension of the action mechanisms explaining the beneficial effects of DHA during an I/R episode, this discovery might have translational beneficial consequences. Indeed, there are many studies conducted to reduce the infarcted area in order to keep the best cardiac function possible. This is mostly tested by infusion of DHA. There are evidences that the preparation of emulsions or pellets of n-3 PUFA in non-controlled oxidative conditions leads to various levels of PUFA oxidation [67]. This means that, if oxidation of PUFA is a prerequisite to obtain a beneficial effect, this effect will vary depending on the quality of the emulsion preparation. By injecting a known



concentration of 4-F<sub>4t</sub>-NeuroP, it will be possible to bypass this problem and reach an immediate effect.

## 5. Conclusion

4-F<sub>4t</sub>-NeuroP, an oxygenated non-enzymatic metabolite of DHA has a direct protective effect on cardiomyocytes in a cardiac rat model of I/R. Administration of 4-F<sub>4t</sub>-NeuroP before ischemia limits infarct size and reduces the occurrence of ventricular arrhythmias. The magnitude of the tissue-sparing effect when 4-F<sub>4t</sub>-NeuroP is administered in prevention indicates that this product may have potential clinical applications in the treatment of acute myocardial infarction by coronary reperfusion. Protective effects seem to be dependent on mitochondria homeostasis through the inhibition of mPTP channels. These data also suggest that 4-F<sub>4t</sub>-NeuroP decreases the pro-apoptotic pathways and limits the liberation of pro-apoptotic factors like cytochrome c.

This study further support the recent findings on the biological effects of 4-F<sub>4t</sub>-NeuroP in the cardiovascular system [22] which concluded that the oxidation of DHA and generation of 4-F<sub>4t</sub>-NeuroP is necessary to prevent ischemia-induced arrhythmias in mouse with myocardial infarction. This discovery bridges the missing relationship between n-3 PUFA consumption and cardiac I/R and shows that 4-F<sub>4t</sub>-NeuroP displayed a unique and unprecedented mode of action in the group of lipid mediators and of any known endogenous biomolecule.

Ultimately, this study opens new perspectives for non-enzymatic oxidized products of n-3 polyunsaturated fatty acids as potent preventive therapeutic way in acute myocardial infarction [68].

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## Conflict of interest

The authors declare that they have no conflict of interest.

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