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Palmitoyl-carnitine increases RyR2 oxidation and sarcoplasmic reticulum Ca\(^{2+}\) leak in cardiomyocytes: Role of adenine nucleotide translocase

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ABSTRACT

Long chain fatty acids bind to carnitine and form long chain acyl carnitine (LCAC), to enter into the mitochondria. They are oxidized in the mitochondrial matrix. LCAC accumulates rapidly under metabolic disorders, such as acute cardiac ischemia, chronic heart failure or diabetic cardiomyopathy. LCAC accumulation is associated with severe cardiac arrhythmia including ventricular tachycardia or fibrillation. We thus hypothesized that palmitoyl-carnitine (PC), alters mitochondrial function leading to Ca\(^{2+}\)-dependent arrhythmia. In isolated cardiac mitochondria from C57Bl/6 mice, application of 10 \(\mu\)M PC decreased adenine nucleotide translocase (ANT) activity without affecting mitochondrial permeability transition pore (mPTP) opening. Mitochondrial reactive oxygen species (ROS) production, measured with MitoSOX Red dye in isolated ventricular cardiomyocytes, increased significantly under PC application. Inhibition of ANT by bongkrekic acid (20 \(\mu\)M) prevented PC-induced mitochondrial ROS production. In addition, PC increased type 2 ryanodine receptor (RyR2) oxidation, S-nitrosylation and dissociation of FKBP12.6 from RyR2, and therefore increased sarcoplasmic reticulum (SR) Ca\(^{2+}\) leak. ANT inhibition or anti-oxidant strategy (N-acetylcysteine) prevented SR Ca\(^{2+}\) leak, FKBP12.6 depletion and RyR2 oxidation/S-nitrosylation induced by PC. Finally, both bongkrekic acid and NAC significantly reduced spontaneous Ca\(^{2+}\) wave occurrences under PC. Altogether, these results suggest that an elevation of PC disturbs ANT activity and alters Ca\(^{2+}\) handling in a ROS-dependent pathway, demonstrating a new pathway whereby altered FA metabolism may contribute to the development of ventricular arrhythmia in pathophysiological conditions.

1. Introduction

In physiological conditions, the high-energy demand required for cardiac function is mainly provided by long-chain fatty acids (FAs). The rate of FAs uptake and oxidation is directly related to the level of circulating FA [1]. As soon as the FAs supply increases, the cardiac efficiency defined as the ratio between the cardiac power and the oxygen consumption drops [2]. In skeletal muscle, under high acute FAs supply, uncoupling protein 3 (UCP3) may export fatty anions from the mitochondrial matrix leading to a reduction of the mitochondrial proton motive force and so of ATP synthesis [3]. In normal heart, UCP3 expression level is low, and FAs-mediating uncoupling has been attributed to the adenine nucleotide translocase (ANT) [4–6]. Before entering into the mitochondria, FAs are first esterified to give fattyacyl-CoA and then the acyl group is transferred to carnitine to form acylcarnitine. Long chain acyl carnitine (LCAC) such as palmitoyl-carnitine (PC) is shuttled in the mitochondria and converted again in acylcarnitine. Long chain acyl carnitine (LCAC) such as palmitoyl-carnitine (PC) is shuttled in the mitochondria and converted again in acylcarnitine. Long chain acyl carnitine (LCAC) such as palmitoyl-carnitine (PC) is shuttled in the mitochondria and converted again in acylcarnitine. Long chain acyl carnitine (LCAC) such as palmitoyl-carnitine (PC) is shuttled in the mitochondria and converted again in acylcarnitine. Long chain acyl carnitine (LCAC) such as palmitoyl-carnitine (PC) is shuttled in the mitochondria and converted again in acylcarnitine. Long chain acyl carnitine (LCAC) such as palmitoyl-carnitine (PC) is shuttled in the mitochondria and converted again in acylcarnitine. Long chain acyl carnitine (LCAC) such as palmitoyl-carnitine (PC) is shuttled in the mitochondria and converted again in acylcarnitine. Long chain acyl carnitine (LCAC) such as palmitoyl-carnitine (PC) is shuttled in the mitochondria and converted again in acylcarnitine.
In addition to energy expenditure, acute application of the long-chain free FA palmitate disturbs Ca\(^{2+}\) handling in healthy cardiomyocytes via a mechanism involving markedly increased reactive oxygen species (ROS) production [16]. Similarly, electro-mechanical dysfunction induced by PC has been linked to an early increase in cellular ROS production [17]. More generally, increased ROS production impairs cellular Ca\(^{2+}\) handling by interfering with a wide range of proteins implicated in excitation–contraction coupling, e.g. the sarcoplasmic reticulum (SR) Ca\(^{2+}\) release channels (type 2 ryanodine receptors, RyR2) [18,19]. RyR2 contains several thiol groups that are highly sensitive to redox modification and, RyR2 oxidation may dissociate the regulatory protein, FKBP12.6, from the channel to promote SR Ca\(^{2+}\) leak [20,21]. Although PC accumulation induces Ca\(^{2+}\) overload and initiates transient inward current [22], whether LCAC affects RyR2 function through redox modification is still unknown.

In the present study we aimed to determine (1) whether acute application of PC on wild type (WT) cardiomyocytes affects Ca\(^{2+}\) handling through redox modification of RyR2 and (2) whether regulation of ANT activity may account for these changes. We found that PC-altered ANT activity leading to an increased mitochondrial ROS production. RyR2 oxidation and SR Ca\(^{2+}\) leak that results in the triggering of cellular Ca\(^{2+}\) waves and ventricular extrasystoles.

2. Material and methods

2.1. Chemicals

Fluo-4 AM, and MitoSOX Red were from Molecular Probes/Invitrogen. Carnitine (C), octanoyl-L-carnitine (OC), palmitoyl-L-carnitine (PC), oleoyl-L-carnitine (OIC), N-acetylcysteine (NAC), bongkrekic acid (BA), and isoprenaline hydrochloride were purchased from Sigma-Aldrich (France). Ap5A was from VWR (France). All compounds were prepared as stock solutions in appropriate solvents. On the day of the experiment, stock solutions were diluted to the desired final concentration in the bath solution. Control solutions contain the same solution of solvent when required (1% dilution of stock solution).

2.2. Isolated mitochondria

Mice hearts were excised and homogenized with the Dounce homogenizer. Then, mitochondria were isolated by differential centrifugation [23]. Mitochondrial protein contents were determined using a b-cation and, RyR2 oxidation may di-
increase, and were approved by the institutional Ethics Committee for Animal Experiments, Languedoc Roussillon (N CEEA-LR-12080). 7-weeks-old C57Bl/6 male mice (Centre d’élevage Janvier, Le Genest Saint Isle, France) were killed by rapid cervical dislocation. Hearts were excised, mounted on the Langendorff apparatus and retrogradely perfused with dissociation buffer contained (in mM): 113 NaCl, 4.7 KCl, 0.6 KH\(_2\)PO\(_4\), 0.6 NaH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 12 NaHCO\(_3\), 10 KHCO\(_3\), 10 Hepes, 30 Taurine (pH 7.4 adjusted with NaOH) and 0.1 mg/ml Liberase TM Research Grade (Roche Diagnostic, Germany). After enzymatic dissociation, hearts were mechanically dissociated in the same solution without enzyme. Cardiomyocytes were then filtered and resuspended in the dissociation buffer where Ca\(^{2+}\) was reintroduced gradually to reach a final concentration of 1 mM Ca\(^{2+}\) [16].

2.3. Confocal imaging

Cells were placed in a bath chamber perfused with a Tyrode solution (in mM): 135 NaCl, 4 KCl, 1.8 CaCl\(_2\), 1 MgCl\(_2\), and 2 HEPES (pH 7.4 adjusted with NaOH) supplemented or not with 10 μM of palmitoyl-L-carnitine (PC10). The bath chamber was placed on the stage of a Zeiss LSM 510 inverted confocal microscope (Zeiss, LePecq France) equipped with a 63× lens (oil immersion, numerical aperture, N.A. = 1.2) allowing the measurement of Fluo4-AM and MitoSOX Red fluorescence.

To measure cytoplasmic Ca\(^{2+}\) transients and spontaneous RyR2 activities (i.e., Ca\(^{2+}\) sparks), isolated cardiomyocytes were loaded for 15 min with the permeant Ca\(^{2+}\) indicator Fluor-AM (3 μM, Molecular Probes), at room temperature. All measurements were performed in line-scan mode (1.5 ms/line), and scanning was carried out along the long axis of the cell. An excitation wavelength of 488 nm was used, and emitted light was collected through a 505 nm long-pass filter. The laser intensity used (3–6% of the maximum) had no noticeable deleterious effect on the fluorescence signal or cell function over the course of the experiment. Ca\(^{2+}\) transient were recorded under field stimulation (10 V at 1 Hz). Analyses were performed using Imagej software. To enable comparisons between cells, fluorescence signals were divided by the minimal fluorescence (F\(_0\)) obtained immediately before the 1 Hz stimulation pulse. The SR Ca\(^{2+}\) content was assessed by measuring the amplitude of cytosolic Ca\(^{2+}\) transients induced by the rapid application of caffeine (10 mM). Spontaneous Ca\(^{2+}\) sparks were recorded in quiescent cells following 5 min stimulations in order to reach steady state SR-Ca\(^{2+}\) content. Ca\(^{2+}\) sparks frequency was analyzed using Imagej software with the Sparksmaster plugin [28].

TMRM was used to measure mitochondrial membrane potential (∆Ψ\(_{in}\)) [29]. Isolated cardiomyocytes were loaded with TMRM (10 nM) for 20 min at room temperature. Confocal images of TMRM fluorescence were obtained by excitation at 568 nm while measuring the emitted light at 585 nm. TMRM fluorescence was measured in five different areas in each cell to minimize the subcellular variability in ∆Ψ\(_{in}\). Images were taken every minute and fluorescence signals were normalized to the fluorescence measured in each cell at the start of the experiment, which was set to 100%. At the end of each experiment, cells were exposed to the mitochondrial uncoupler FCCP (10 nM) to determine the dynamic range of the dye.

Mitochondrial ROS production was measured using MitoSOX Red dye. Cardiomyocytes were incubated 45 min at 37 °C with MitoSOX Red [16]. Cells were perfused with the Tyrode solution and field stimulated during 5 min to reach a steady state. Then the PC solution was perfused during 15 min. x-y confocal images of the emitted light at 585 nm were recorded every 2 min intervals by excitation at 488 nm. The first acquisition was done after 1 min of Tyrode perfusion. Fluorescence changes, measured after 10 min of PC application, were
normalized to the steady state values recorded after 5 min of Tyrode perfusion (Fig. 3B).

2.5. Cellular arrhythmia

Cellular arrhythmias, such as abnormal Ca\(^{2+}\) waves, were quantified with the IonOptix\(^\text{®}\) system (Hilton, USA) \[30,31\]. Cardiomyocytes were loaded with the ratiometric dye 30 min at RT with 10 \(\mu\)M indo-1 AM (Invitrogen) and perfused with a Tyrode solution containing 1 mM isoprenaline. Cardiomyocytes were field-stimulated at 3 Hz (20 V, 1 ms), and simultaneously illuminated at 305 nm using a xenon arc bulb light. Indo-1 AM fluorescence emitted at 405 nm and 480 nm was recorded simultaneously using IonOptix\(^\text{®}\) acquisition software (Hilton). To record spontaneous Ca\(^{2+}\) waves in the resting condition, stimulation was stopped for 30 s once Ca\(^{2+}\) transient steady state was reached.

2.6. RyR2 biochemistry

Using Langendorff technique, the whole heart was perfused 10 min with Tyrode’s solution containing or not PC10 and/or BA. Atria and right ventricles were then excised and the left ventricles were quickly frozen. Left ventricle was lysed in 1 ml of a buffer containing (in mM) 10 Tris-maleate (pH 6.8), 35 NaF, 1% Triton and a cocktail of protease inhibitors (Roche 11873580001). An anti-RyR antibody was used to immunoprecipitate RyR2 from 500 \(\mu\)g of left ventricular homogenate. Samples were incubated with an anti-RyR antibody in 0.5 ml of a modified RIPA buffer (10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Triton; 5 mM NaF and protease inhibitor cocktail) for 2 h at 4 \(^\circ\)C. The immune complex was incubated with protein A/G magnetic beads (Pierce 88802) at 4 \(^\circ\)C for 2 h, after which the beads were washed out three times with RIPA buffer. To detect RyR2 protein oxidation, the immune complex was treated with 2.4 \(\mu\)M dinitro-phenylhydrazine (DNPH) and the DNPtran derivatized carbonyls were detected using on Oxyblot Protein oxidation detection Kit (Millipore S7150). Proteins were separated on SDS/PAGE gels and transferred onto nitrocellulose membranes for 1 h at 100 V. The immunoblots were prepared using antibodies against RyR (1:1000), anti Cys-NO antibody (Sigma-Aldrich N5411, 1:1000), anti-DNPH (1:300) and anti-FKBPI2.6 (RD System AF 4174, 1:1000). All immunoblots were developed and quantified using the Odyssey infrared imaging system (LICOR Biosystems) and infrared-labeled secondary antibodies.

2.7. Electrocadiogram recording

Mice were equipped of subcutaneous implantable devices (PhysioflareTAE-F10 model, Data Sciences International, USA). Surgical implantation of ECG devices was realized under general gaseous anesthesia (2.5% isoflurane/O\(_2\), Iso-vet, Piramal Healthcare UK) and monitoring, on retro-controlled heating pad. Lidocaine was solely disposed on the wound after surgery during 2 days of recuperation.

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**Fig. 1.** PC10 affects ADP/ATP translocase activity at a non-saturating ADP concentration (6.5 \(\mu\)M). (A) Typical example of changes of NADPH fluorescence that reflects ANT activity \((\lambda_{\text{ex}} 360 \text{ nm}, \lambda_{\text{em}} 465 \text{ nm})\). The test was performed in different conditions: non-treated mitochondria (NT, square), mitochondria incubated with Ap5A (circle), BA (open triangle), or Ap5A + BA (filled triangle). (B) The ANT activity is estimated using the area under the curve in the different conditions normalized to the activity of non-treated mitochondria. (C) The application of PC10, or PC10 + Ap5A decreases ANT activity. Experiments were performed in triplicate. Statistical differences between NT and other groups *\(p < 0.05\); (n = 6).

**Fig. 2.** PC10 does not modify the mPTP opening. (A) Typical example of an experiment showing the decrease in optical density reflecting the mPTP opening. The optical density measured at 543 nm of mitochondria was followed in non-treated mitochondria (square), in presence of PC10 (open star) or in the presence of different concentrations of Ca\(^{2+}\), 6 \(\mu\)M (open triangle), 25 \(\mu\)M (filled triangle) or 50 \(\mu\)M (filled circle). (B) Summarizes the mean results from 5 different experiments performed in duplicate. Different PC concentrations were applied with Ap5A. As for PC, BA alone did not induce mPTP opening. Statistical differences between NT and other groups; *\(p < 0.01\); and **\(p < 0.001\) (n = 6).
and experiments began one week after surgery. ECGs were recorded using IOX and analyzed with ECG auto software (EMKA technologies, France). Heart rate, PR, QRS, and QT intervals were measured during the 6 h after 100 μL intravenous (IV) injection of a physiological serum solution (control) or after PC10. Experiments were performed in the same mice after one day of clearance. QT interval was defined between the first deviations from an isoelectric PR interval until the return of the ventricular repolarisation to the isoelectric TP baseline. This method included in the measure the low-amplitude portion of the T-wave and allows a complete ventricular repolarization of ventricles. The QT correction was performed with the adapted Bazett’s formula of Mitchell. The triggering of spontaneous arrhythmia as single or in salvos ventricular extrasystoles was counted after control or PC10 challenge. The housing, the recording and the analyses of ECG respected the Lambeth convention.

2.8. Statistical analysis

Data are presented as mean ± SEM. Statistical significance was defined as *p < 0.05, **p < 0.01, and ***p < 0.001 using Student’s t test (paired or unpaired) or analysis of variance (one- or two-way), followed by a Bonferroni selected-comparison test. The n represents the number of cells and N the numbers of animals studied.

3. Results

3.1. Effects of palmitoyl-carnitine on ANT activity

Depending on the FAs nature, ANT activity is regulated differentially. For instance, free FAs activates ANT whereas palmitoyl-CoA inhibits it [5]. Here we first determined whether PC form would affect ANT activity. We used an ADP/ATP translocase assay on ventricular isolated mitochondria as previously described [23]. This assay is based on

![Graph showing the decrease in SR Ca^2+ content and Ca^2+ transient amplitude with PC10 application.](image)

**Fig. 4.** PC10 decreases SR Ca^2+ content and Ca^2+ transient amplitude. (A) Representative ΔF/F₀ signal recorded in non-treated (NT) or incubated with PC10. (B) Mean value of SR Ca^2+ content. (C) Ca^2+ transient amplitude or (D) decay time constant (τ) of Ca^2+ transient signal obtained in isolated ventricular cardiomyocytes treated or not with PC10, 10 μM carnitine (C10), and 10 μM octanoylcarnitine (OC10). Cardiomyocytes were pre-incubated 15 min with NAC or DPI. **p < 0.01; and ***p < 0.001 compared to the same conditions prior to application of PC10 (n = 9–12 cardiomyocytes in each condition).
NADPH fluorescence measurement upon ADP application (6.5 μM). The subsequent NADPH formation depends on both the adenylate kinase 2 (AK2) and the ANT activities. We thus used diadenosinepentaphosphate (Ap5A, 10 μM) and bongkrekic acid (BA, 20 μM), specific inhibitors of AK2 and ANT respectively in order to differentiate both activities (Fig. 1 A) [23]. In the presence of 2.5 μM PC (PC2.5) NADPH fluorescence, with or without Ap5A, was unchanged whereas 10 μM PC (PC10) decreased NADPH formation in presence or in absence of Ap5A (Fig. 1B). In the presence of BA, PC2.5 and PC10 were ineffective, indicating that PC10 specifically decreased the ATP/ADP translocase activity without affecting AK2.

Under stress conditions, ANT associated with other mitochondrial proteins, such as cyclophilin D, forms the mitochondrial permeability transition pore (mPTP) [32]. To determine whether PC affects mPTP formation, we performed a mitochondrial-swelling test [24] (Fig. 2). In the presence of PC2.5 or PC10, the absorbance was unchanged whereas application of Ca2+ (50 μM) as a positive control induced a drop of optical density. Altogether these results show that PC10 modulates ANT activity without affecting mPTP opening.

3.2. Effects of palmitoyl-carnitine on mitochondrial ΔΨm and ROS production

To estimate the uncoupling effects of PC10, we measured mitochondrial membrane potential (ΔΨm) using TMRE on intact ventricular cardiomyocytes. FCCP (10 μM) was applied at the end of each experiments to fully uncouple and dissipate ΔΨm. (Fig. 3A) PC10 application decreases TMRE fluorescence by ~20% whereas in the presence of BA, PC10 was ineffective (Fig. 3A), indicating that BA prevents uncoupling mediated by PC10 [4,6]. We next measured mitochondrial ROS production using MitoSOX Red on intact ventricular cardiomyocytes [16]. Application of PC10 significantly increased mitochondrial ROS production by ~20% (122.4 ± 6.4 vs. 101.6 ± 0.9; Fig. 3C, D). In the presence of the ANT inhibitor, BA (20 μM), PC10 was unable to increase mitochondrial ROS production (Fig. 3D). When applied with the mPTP inhibitor, cyclosporine A (CsA, 0.1 μM), PC10 significantly increased MitoSOX Red fluorescence to the same extent than in the absence of CsA (Fig. 3D). These results indicate that PC increases mitochondrial ROS production through its effects on ANT and independently of mPTP.

3.3. Effects of palmitoyl-carnitine on Ca2+ transients

PC has been reported to increase intracellular Ca2+ level on quiescent cardiomyocytes, however the effects of PC on triggered Ca2+ transients is unknown [33]. In intact isolated ventricular cardiomyocytes, application of PC10 significantly decreased Ca2+ transient amplitude (Fig. 4A, C) as well as SR Ca2+ content (Fig. 4B). The use of carnitine alone (10 μM) or a middle chain fatty acid, octanoyl-carnitine (10 μM) did not affect Ca2+ transient amplitude. This result suggests that the decrease of Ca2+ transient amplitude is specific to PC. NAC (20 mM), a broad-spectrum anti-oxidant, prevented PC10-induced Ca2+ transients decrease, whereas DPI (diphenyleneiodonium), an inhibitor of NADPH oxidase (NOX) was ineffective (Fig. 4B). Although, PC10 decreased Ca2+ transients’ amplitude, the Ca2+ transients’ decay was not affected (Fig. 4D). To summarize, these results indicate that LCAC affect SR release of Ca2+ through an increased ROS production independently of NOX activity.

3.4. Effects of palmitoyl-carnitine on SR Ca2+ leak

We next determined whether PC10 affects RyR2 activities by measuring the SR spontaneous Ca2+ release events (Ca2+ sparks) using confocal microscopy. In intact isolated ventricular cardiomyocytes, application of PC10 significantly increased Ca2+ sparks frequency reflecting a SR Ca2+ leak (Fig. 5A, B) whereas in the presence of BA (20 μM), PC10 was ineffective (Fig. 5B). Similarly, when cardiomyocytes were incubated with the non-specific antioxidant, NAC (20 mM), PC10 did not affect Ca2+ sparks frequency (Fig. 5A, B). It is to note that BA and NAC alone did not affect calcium sparks frequency. In addition sparks frequency was not affected by carnitine or octanoyl-carnitine (Fig. 5C). These results indicate that LCAC only induce SR Ca2+ leak via ANT activity and ROS production.

Increased oxidation state of RyR2 may account for SR Ca2+ leak [18, 21]. Therefore, we assessed RyR2 post-translational modification in left ventricle from heart perfused for 10 min with either PC10 alone or
3.5. Effects of palmitoyl-carnitine on spontaneous Ca\(^{2+}\) waves and ventricular arrhythmia

In pathological conditions, an increased SR Ca\(^{2+}\) leak is known to initiate Ca\(^{2+}\) waves and to trigger arrhythmic events, originating from delayed after depolarization [34]. To investigate the propensity of intact ventricular cardiomyocytes to generate spontaneous Ca\(^{2+}\) waves in the presence of PC10, we field-stimulated cardiomyocytes, loaded with the Ca\(^{2+}\) indicator indo-1-AM, during 30 s at 3 Hz frequency. Spontaneous Ca\(^{2+}\) waves were detected during a subsequent resting period (30 s). While in control conditions, the diastolic Ca\(^{2+}\) remained stable during the rest period, after PC10 application, about 70% of the cardiomyocytes triggered Ca\(^{2+}\) oscillations and/or waves, indicative of an arrhythmic behavior (69.3 ± 3.6%, Fig. 7B). Confocal microscopy experiments show that PC10-induced Ca\(^{2+}\) waves originate from an increase in Ca\(^{2+}\) sparks events (Fig. 7C). Again, when cardiomyocytes were incubated with BA or NAC, PC10 failed to trigger spontaneous Ca\(^{2+}\) events (Fig. 7B). PC10 propensity to induce arrhythmic events was further studied in vivo by telemetric approach (Fig. 8). Although IV injection of PC10 did not significantly impact basal ECG parameters (Fig. 8A–E), PC10 increased the occurrence of ventricular extrasystoles (Fig. 8F–G). In addition we also observed in 2 out of 6 PC10 treated mice the triggering of non-sustained ventricular tachycardia as defined by the Lambeth conventions [35], whereas untreated mice did not (Chi2 = 0.12; Fig. 8H). None of the untreated and treated PC10 mice developed sustained ventricular tachycardia.

4. Discussion

LCAC level varies depending on the metabolic state and may increase under pathophysiological conditions. In the present study, we...
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Fig. 7. PC10 increases the occurrence of cellular Ca\(^{2+}\) waves. Variation of cytosolic Ca\(^{2+}\) of isolated cardiomyocytes was followed during the field stimulation and rest periods. (A) Representative trace obtained in control (NT) and in the presence of PC10, NAC + PC10 and BA + PC10 perfusion. (B) Results are presented as the percentage of cells having diastolic Ca\(^{2+}\) waves. Variation of cytosolic Ca\(^{2+}\) waves observed, under confocal microscopy, in presence of PC10. Each cell is its own control. NT (n = 28), PC10 (n = 28), NAC + PC10 (n = 19), BA + PC10 (n = 20) ***p < 0.001 compared to non-treated conditions in each group.

Fig. 1, associated with ΔΨm dissipation (Fig. 3A and [4,6]) and mitochondrial ROS production (Fig. 3D). Conversely, bongkrekic acid, which inhibits both the uncoupling and the ATP\(^{4+}\)/ADP\(^{3−}\) transport (Fig. 1; [4,6]), prevents ΔΨm and mitochondrial ROS production.

A cellular elevation of ROS leads to the oxidation of numerous proteins resulting in myocardial dysfunction [41]. Among ROS potential targets, calcium-handling proteins are particularly sensitive to redox modulation [18]. RyR2 is highly sensitive to redox state due to the large number of cysteines that composed the homo-tetramer. The nature and the number of oxidized and/or S-nitrosylated cysteine residues directly impact on the channel properties. Indeed, a RyR2 monomer contains 89 cysteine residues and 2 per FKBP12.6 protein, and it was estimated that around 20 cysteine residues per subunit are free (80 per tetrameric RyR2). It was also estimated that channel activation requires a poly-S-nitrosylation where 3 sites per subunit (∼11 per tetramer) induced a maximal RyR2 activation, whereas ∼2 sites per RyR2 did not significantly affect RyR2 open probability. Meaning that a low level of RyR2 S-nitrosylation per se does not affect significantly RyR2 function. On the other hand, oxidation of >7 thiols per subunit induces an irreversible activation of the channel through disulfide bonds formation between RyR2 subunits [42–44]. Irreversible RyR2 oxidation unambiguously increases RyR2 open probability and SR Ca\(^{2+}\) leak, however the level of RyR2 S-nitrosylation, which is reversible, has been proposed to increase or decrease RyR2 open probability (for review see [45,46]). Indeed, more than S-nitrosylation demonstrate that PC-induced decrease in ANT activity causes an increased mitochondrial ROS production and consequently RyR2 oxidation and S-nitrosylation, and FKBP12.6 dissociation. These RyR2 post-translational modifications trigger SR Ca\(^{2+}\) leak and promotes the occurrences of diastolic Ca\(^{2+}\) waves and arrhythmic events.

In physiological conditions, the heart is preferentially fuelled with FAs. However, compared to glucose, an increase in FAs consumption reduces cardiac efficiency. Among the mechanisms involved, a modulation of ANT has been suggested [2]. Indeed, increased palmitoyl-CoA content inhibits ANT both from the extra-mitochondrial side and the mitochondrial matrix [36]. Altered ANT would reduce the ATP/ADP turnover and subsequently the ATP synthase activity. Consequently, it would increase mitochondrial ROS production from the electron transport chain [37]. Accordingly, PC-induced ROS production has been ascribed to an incomplete blockade of the respiratory chain [38]. In the present study the decrease in ANT activity induced by PC is presumably due to its conversion into palmitoyl-CoA rather than a direct effect, as LCAC does not inhibit ANT on submitochondrial particles [5,39,40]. In addition, free fatty acids have been proposed to uncouple mitochondria via the ANT either through an allosterical stimulation of H\(^{+}\) transfer or through the translocation of fatty acid under their anionic form. Alternatively, fatty acid anions could also increase negative surface charges facilitating H\(^{+}\) transfer through the ANT [2]. Consequently, an increased uncoupling through the ANT may compete with the ATP\(^{3−}\)/ADP\(^{3−}\) translocation capacity of the ANT as reported in
or oxidation itself, the nitroso/redox balance is critical for the modulation of RyR2 function. In physiological conditions, RyR2 is endogenously S-nitrosylated at a low level, which does not affect the RyR2 open probability. Nevertheless, a decrease in the basal S-nitrosylation level increases the number of free cysteines available for oxidation. Consequently, the RyR2 oxidation may increase which leads to SR Ca\(^{2+}\) leak [47,48]. In this context RyR2 S-nitrosylation competes with the RyR2 oxidation level and is considered as a protective reaction to counteract an irreversible thiol oxidation of the RyR2. On the other hand, in pathophysiological conditions where NO homeostasis is altered and the number of S-nitrosylated cysteine is substantially increased, SR Ca\(^{2+}\) leak is enhanced [31,46]. However, in pathological conditions, the nitroso/redox balance was not systematically measured and a concomitant increase in oxidation may also contribute to SR Ca\(^{2+}\) leak [31]. In the present study, increase S-nitrosylation might be a compensatory mechanism to limit the extent of thiol oxidation or might synergistically contribute to altered Ca\(^{2+}\) release during systole.

Normal closing of RyR2 in diastole is a key parameter, which prevents Ca\(^{2+}\) leak and subsequent SR Ca\(^{2+}\) depletion, thereby preserving appropriate conditions for an optimal Ca\(^{2+}\) release during systole. Increased RyR2 oxidation and/or S-nitrosylation are associated with both a decrease in FKBP12.6 binding affinity to the channel and an increase RyR2 Ca\(^{2+}\) sensitivity [20,21]. Therefore, disruption of the RyR2–FKBP12.6 interaction increases diastolic SR Ca\(^{2+}\) leak through increased RyR2 activity [50]. As a matter of fact, PC-induced RyR2 oxidation and S-nitrosylation disturb diastolic Ca\(^{2+}\) homeostasis with decreased peak Ca\(^{2+}\) transients and triggering of ectopic Ca\(^{2+}\) waves. Increased diastolic SR Ca\(^{2+}\) leak and Ca\(^{2+}\) waves destabilize secondary resting membrane potential and triggered ectopic action potential [51]. When SR Ca\(^{2+}\) leak exceed the SR Ca\(^{2+}\) uptake capacity, Ca\(^{2+}\) extrusion from the Na\(^{+}~/\text{Ca}^{2+}\) exchanger is increased, generating an inward current [51]. In the mean time, the increased SR Ca\(^{2+}\) leak decreases the outward component of the inward rectifying potassium current (I\(_{\text{K1}}\)) [52,53]. The increased inward Na\(^{+}~/\text{Ca}^{2+}\) exchanger current and reduced outward I\(_{\text{K1}}\) synergistically contribute to the genesis of delayed afterdepolarizations [51–53]. Accordingly PC has been reported to trigger transient inward current and delayed afterdepolarization in adult ventricular cardiomyocytes [22]. Moreover, NAC treatment reduces RyR2 oxidation, S-nitrosylation and prevents RyR2 SR Ca\(^{2+}\) involvement of these specific cysteine residues in the displacement of the FKBPs under oxidation and/or poly-S-nitrosylation is still speculative, these results reinforce the hypothesis that RyRs/ FKBP interactions in heart and skeletal muscle might be directly sensitive to oxidation and/or poly-S-nitrosylation [20,21].
leak as previously reported [31]. This confirms the deleterious role of ROS on Ca2+ handling and subsequent arrhythmic events [29,47,54]. In addition, we showed that the blockade of ANT activity by BA reduces PC10 effects on RyR2. These results reveal a direct link between ANT dysfunction, ROS production and RyR2 function. Although in the present study PC10 application did not significantly affect Ca2+ transients decay, cumulative deleterious effects of PC on other Ca2+ handling proteins could not be excluded [55].

The uptake of cardiac FAs is directly correlated to the plasmatic level of non-esterified FAs [56]. Under metabolic stress, plasmatic FA concentration increases dramatically as well as FAs covalently bound to coenzyme A or carnitine [13,51,57]. For instance a rapid increase of plasmatic FA concentration in diabetic cardiomyopathy rats is correlated with a four-fold increase of LCAC in myocardium [57] and mitochondrial dysfunction during metabolic syndrome is associated with arrhythmogenic events [58]. Moreover, increased levels of long chain FAs have also been suspected to be involved in unexplained sudden death in infants with conduction defects or ventricular fibrillation and tachycardia [59]. In addition to LCAC accumulation, acute ischemia is also associated with a decreased ANT activity, independently of any change in oxidative phosphorylation, [60–62] and an increased RyR2 oxidation leading to reperfusion injury [63]. Similarly, PC level was shown to correlate with NVHA classification and high plasma levels of PC were significantly associated with serious adverse events and poor prognostic of heart failure patients [64]. Finally, decreased ANT function per se triggers a progressive cardiomyopathy and elicits arrhythmic events [15,65–67] associated with mitochondrial ROS production [65,68].

To conclude growing evidences demonstrate a link between altered FAs acids metabolism and arrhythmia in various pathological and stress conditions in both animal model and human. The present study proposes a novel model whereby mitochondrial ANT-induced ROS production is central for FA-induced arrhythmias. Strategies based on a re-equilibration of mitochondrial function could be promising to limit PC accumulation and subsequent fatal arrhythmias in a wide range of pathology [12,69].

Disclosures
None.

Transparency Document
The Transparency document associated with this article can be found in the online version.

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