Biochimica et Biophysica Acta 1852 (2015) 749–758

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

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

J. Roussel [a], J. Thireau [a], C. Brenner [b], N. Saint [a], V. Scheuermann [a], A. Lacampagne [a], J.-Y. Le Guennec [a], J. Fauconnier [a,*]

[a] INSERM U1046, UMR CNRS 9214, Université de Montpellier, Montpellier, France
[b] INSERM UMR-S769 LabEx LERMIT, Université Paris-Sud, Châtenay-Malabry, France

ARTICLE INFO

Article history:
Received 19 September 2014
Received in revised form 16 January 2015
Accepted 18 January 2015
Available online 23 January 2015

Keywords:
Long chain acyl carnitine
Reactive oxygen species
ANT
RyR2

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 μM 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 μM) 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 fatty-acyl-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 fatty-acyl-CoA to be β-oxidyzed [7]. Changes in FA metabolism affect the LCAC levels and the concentration increases from 2–6 μM to 10–30 μM during diabetic cardiomyopathy, genetic disorders or ischemic heart failure [8–11]. LCAC accumulation contributes to the ventricular dysfunctions as ventricular extrasystole, tachycardia and ventricular fibrillation [12–14]. Similarly, cardiac ANT deficiency in human is associated with ventricular arrhythmia [15]. Although a decrease in LCAC accumulation is known to reduce ventricular arrhythmias, the mechanisms whereby LCAC induces ventricular arrhythmia remain elusive.

Abbreviations: AK2, adenylate kinase 2; ANT, adenine nucleotide translocase; Ap5A, P1P5-diadenosine-5′-pentaphosphate; BA, bongkrekic acid; C, carnitine; CsA, cyclosporine A; DPI, diphenyleneiodonium; FA, fatty acid; FKBP12.6, FK506 binding protein 12.6 kDa; I_{K1}, inward rectifying potassium current; IV, intravenous; LCAC, long chain acyl carnitine; mPTP, mitochondrial permeability transition pore; NAC, N-acetylcysteine; NOX, NADPH oxidase; NT, non-treated; OC, octanoyl-L-carnitine; OlC, oleoyl-L-carnitine; PC, palmitoyl-carnitine; ROS, reactive oxygen species; RyR2, type 2 ryanodine receptor; SR, sarcoplasmic reticulum; UCP3, uncoupling protein 3; WT, wild type.

* Corresponding author at: Inserm U1046, UMR CNRS 9214, Physiologie & Médecine Expérimentale, Cœur et Muscles, CHU Arnaud de Villeneuve, 371 Rue du doyen G. Giraud, 34295 Montpellier, France. Tel.: +33 4 67 41 52 22; fax: +33 4 67 41 52 42.
E-mail address: jeremy.fauconnier@inserm.fr (J. Fauconnier).

http://dx.doi.org/10.1016/j.bbadis.2015.01.011
0925-4439/

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 thiols 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 (OlC), 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 micro BCA assay (BCA Protein Assay Kit, Thermo Fisher Scientific).

In order to measure mitochondrial permeability transition pore (mPTP) opening, isolated heart mitochondria were diluted in 200 μl of hypo-osmotic buffer composed of (in mM): 200 saccharose, 5 succinate, 10 MOPS, 0.01 EGTA, 1 H_3PO_4 (pH adjusted at 7.4), and incubated with different PC concentrations. mPTP opening was estimated by spectrophotometry where the decrease of optical density at 540 nm reflects the mitochondrial swelling. Different Ca^{2+} concentrations (6, 25, 50 μM) were used as positive control [24]. The effects of different treatments were normalized according to the following equation (NT − X) / (NT − Ca^{2+} 50 μM) ∗ 100, where NT is the value obtained in non-treated mitochondria, X is the value obtained with the test condition, and "Ca^{2+} 50 μM" is the value obtained with 50 μM of Ca^{2+}.

A non-radioactivity assay was used to estimate ANT activity [25]. Briefly, the ADP/ATP exchange rate was evaluated by following NADPH fluorescence (λexc: 360 nm, λem: 465 nm) in the presence of 6.5 μM external ADP and 2.5 mM glucose, 1 E.U. hexokinase, 0.2 mM NADPH, 0.5 E.U. glucose-6-phosphate dehydrogenase. An increase of NADPH fluorescence reflects an increase of ANT activity as previously described [26,27]. 10 μM of P1P5-diadenosine-5′-pentaphosphate (Ap5A) was used to determine the influence of adenylate kinase-dependent ATP synthesis.

2.3. Cell isolation

All procedures conformed to European Parliament Directive 2010/63/EU and the 22 September 2010 Council on the protection of animals, 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_2PO_4, 0.6 Na_2HPO_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].

2.4. 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 Fluo4-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 fluorescent (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 ($\Delta\Psi_m$) [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 $\Delta\Psi_m$. 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 μM) 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