Acute exercise protects against calcium-induced cardiac mitochondrial permeability transition pore opening in doxorubicin-treated rats

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ABSTRACT

The use of DOX (doxorubicin), an antibiotic used in oncological treatments, is limited by a dose-related cardiotoxicity against which acute exercise is protective. However, the mitochondrial-related mechanisms of this protection remain unknown. Therefore the present study aimed to determine the effects of an acute endurance exercise bout performed 24 h before DOX treatment on heart and liver mitochondrial function. A total of 20 adult male Wistar rats were divided into groups as follows: non-exercised with saline (NE + SAL), non-exercised DOX-treated (NE + DOX), exercised with saline (EX + SAL) and exercised DOX-treated (EX + DOX). The animals performed a 60 min exercise bout on a treadmill or remained sedentary 24 h before receiving either a DOX bolus (20 mg/kg of body weight) or saline. Heart and liver mitochondrial function [oxygen consumption, membrane potential (∆Ψ) and cyclosporin-A-sensitive calcium-induced MPTP (mitochondrial permeability transition pore) opening] were evaluated. The activities of the respiratory complex, Mn-SOD (superoxide dismutase), caspases 3 and 9, as well as the levels of ANT (adenine nucleotide translocase), VDAC (voltage-dependent anion channel), CypD (cyclophilin D), Bax and Bcl-2, were measured. Acute exercise prevented the decreased cardiac mitochondrial function (state 3, phosphorylative lag-phase; maximal ∆Ψ generated both with complex I- and II-linked substrates and calcium-induced MPTP opening) induced by DOX treatment. Exercise also prevented the DOX-induced decreased activity of cardiac mitochondrial chain complexes I and V, and increased caspase 3 and 9 activities. DOX administration and exercise caused increased cardiac mitochondrial SOD activity. Exercise ameliorated liver mitochondrial complex activities. No alterations were observed in the measured MPTP and apoptosis-related proteins in heart and liver mitochondria. The results demonstrate that acute exercise protects against cardiac mitochondrial dysfunction, preserving mitochondrial phosphorylation capacity and attenuating DOX-induced decreased tolerance to MPTP opening.

Key words: adriamycin, apoptosis, doxorubicin (DOX), exercise, mitochondrial bioenergetics, mitochondrial permeability transition pore (MPTP).

Abbreviations: ∆Ψ, mitochondrial membrane potential; ANT, adenine nucleotide translocase; cTnI, cardiac troponin I; CypD, cyclophilin D; DCIP, 2,6-dichlorophenolindophenol; DOX, doxorubicin; EX, acutely exercised; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase; IME, internyomyofibrillar; MPTP, mitochondrial permeability transition pore; NE, non-exercised; pNA, p-nitroaniline; RCR, respiratory control ratio; SAL, saline; SOD, superoxide dismutase; SS, subsarcolemmal; TPP+, tetraphenylphosphonium; VDAC, voltage-dependent anion channel.

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INTRODUCTION

DOX (doxorubicin), also known as adriamycin, is a highly effective antibiotic used to treat several types of cancer. Unfortunately, the clinical use of DOX is limited by the occurrence of a dose-related cardiac toxicity that results in life-threatening cardiomyopathy. Previous studies have described that DOX-induced cardiomyocyte dysfunction is associated with increased levels of oxidative damage and apoptosis involving mitochondria [1,2]. The myocardial toxicity of DOX is related to a redox-cycling process at mitochondrial complex I, which leads to increased oxidative stress, depletion of cellular reducing equivalents, inhibition of mitochondrial respiration and phosphorylation, and interference with cellular calcium homoeostasis [2,3]. Since the cardiomyocyte is an energy-demanding tissue, mitochondria are important players in cardiac bioenergetics and hence it is considered that the toxicity of DOX at the mitochondrial level explains, in part, the development of the cardiotoxicity associated with drug treatment.

One sensitive and early marker of DOX-induced cardiotoxicity is a loss of calcium-loading capacity by mitochondria. Rather than inhibiting calcium uptake, DOX stimulates calcium release from the mitochondrial matrix through induction of the MPTP (mitochondrial permeability transition pore) [4–9], a physiological mitochondrial channel, in a manner dependent on calcium overload and oxidative stress. In fact, the oxidative stress caused by DOX on cardiac mitochondria and the associated disturbances in cellular calcium homeostasis are considered an optimal environment for the formation and opening of the MPTP, which contributes to a mitochondrial bioenergetic failure [1,2]. In an opened state, the MPTP is also associated with the release of proapoptotic mitochondrial proteins, such as cytochrome c or apoptosis-inducing factor, into the cytoplasm where they contribute to trigger cell death. Depending on the treatment regimen, DOX administration can also cause perturbations in liver mitochondrial bioenergetics, although the severity is doubtless lower than in the heart [4,10].

Physical exercise, particularly endurance training, has been importantly advised as a non-pharmacological tool against myocardial injury. In fact, it is well described that endurance exercise training improves myocardial tolerance to deleterious stimuli that cause intracellular oxidative stress and apoptosis [11,12]. Many preventive and therapeutic strategies have been explored to counteract DOX toxicity and dysfunction, such as antioxidant supplementation and exercise. The benefits of chronic aerobic exercise against DOX-induced cardiac toxicity and dysfunction have been established already. Previous work has suggested that the advantage of endurance training on the cardiovascular system of DOX-treated rats include the protection of cardiac tissue and mitochondria against increased oxidative damage and apoptosis [11,13–20]. It is also known that acute exercise is cardioprotective against damaging insults; in fact, studies revealed that a single endurance exercise bout preserved cardiac function and protected the heart against ischaemia/reperfusion-induced oxidative damage and reduced infarct size [21–24]. Recently, Wonders et al. [25] reported that an acute exercise bout performed 24 h before DOX treatment protected against cardiac dysfunction and decreased lipid peroxidation. However, the mechanisms behind this protection, particularly those involving mitochondria, were not described and remain elusive. Ji and Mitchel [26] reported that DOX administration after a single bout of acute exhaustive exercise antagonized the increased heart mitochondrial respiration induced by the exercise during both ADP-stimulated and basal respiration. However, the effects of prior acute endurance exercise on the susceptibility of cardiac mitochondria to form the MPTP and cells to undergo apoptosis in the DOX-treated rats was never explored. Considering that the loss of calcium loading capacity is an early and sensitive marker of DOX-induced cardiotoxicity we tested whether a bout of acute exercise attenuates the loss of cardiac mitochondrial calcium-loading capacity after DOX treatment. Isolated liver mitochondria from the different experimental groups were also used in order to verify whether the DOX and/or exercise effects were organ specific. The main finding of the present study was that acute exercise protected against DOX-induced cardiac mitochondrial dysfunction by increasing calcium accumulation capacity, thus reducing the DOX-induced increased susceptibility to calcium-induced MPTP opening.

MATERIALS AND METHODS

Animals

Male Wistar rats (aged 6–8 weeks and weighing 200 g at the beginning of the treatments) were obtained from Charles River. During the experimental protocol, animals were housed in collective cages (two rats per cage) and were maintained in a room under normal conditions (21–22 °C and ~50–60% humidity) in 12 h light/12 h dark cycles, receiving food and water ad libitum. Rats were randomly assigned into four groups (n = 5 per group): non-exercised with saline (NE + SAL), non-exercised receiving DOX (NE + DOX), acutely exercised with saline (EX + SAL) and acutely exercised treated with DOX (EX + DOX). The sample size of the present study resulted from the low dispersion and high consistency of the data observed during the experiments. The local Institutional Review Board approved the experimental protocol, which was in compliance with the Guidelines for Care and Use of Laboratory Animals in research.
Acute exercise protocol
The protocol of animal acclimatization and acute exercise followed that recently used by Wonders et al. [25]. Briefly, animals from the exercised groups were accustomed to treadmill exercise over 2 weeks. During the first week, animals ran 10 min/day for 3 days/week at a 10 m/min speed (0 % gradient). During the second week, rats ran 10 min/day for 3 day/week at 20 m/min speed (0 % gradient). This acclimatization protocol has been shown to have minimal effect on mitochondrial enzymes [27]. At 24 h after the acclimatization protocol, rats successfully performed an acute single bout of treadmill running of 60 min divided into three phases: phase 1 (min 0–5) where animals ran at 15 m/min and at 0 % gradient; phase 2 (min 5–10) where animals ran at 23 m/min on a 0 % gradient; and phase 3 (min 10–60) where animals ran at 25 m/min on a 5 % gradient. The animals from non-exercised groups were placed on a non-moving treadmill for 60 min such that they were exposed to potential handing and environment stresses induced by the treadmill itself.

DOX administration
At 24 h after the cessation of the acute endurance exercise bout, or the control non-exercised period, animals from the NE + DOX and EX + DOX groups received a single intraperitoneal DOX dose (20 mg/kg of body weight), whereas animals from the NE + SAL and EX + SAL groups were injected with 0.5 ml of vehicle (sterile saline solution).

Animal plasma and heart extractions
At 5 days after the DOX or saline treatments, animals were anaesthetized with sodium pentobarbital (50 mg/kg of body weight) and placed in the supine position. After that, the abdominal cavity was opened to expose the inferior cava vein and a blood sample of approx. 2 ml was collected in an EDTA-containing tube. The blood was immediately centrifuged (5000 g for 5 min at 4 °C) and an aliquot of plasma was obtained and stored at −80 °C for biochemical determination of cTnI (cardiac troponin I) levels, and GOT (glutamate oxaloacetate transaminase) and GPT (glutamate pyruvate transaminase) activities. After a quick opening of the chest and abdominal cavities, rat hearts and livers were then rapidly excised, rinsed, carefully dried and weighed. A portion of approx. 20–25 mg of tissue was separated, homogenized in homogenization buffer [20 mM (100 mg/ml) Tris/HCl, pH 7.4] using a Teflon pestle on a motor-driven Potter–Elvehjem glass homogenizer at 0–4 °C and the resulting supernatant was centrifuged at −80 °C for later determination of biochemical parameters.

Isolation of rat heart and liver mitochondria
Mitochondria were prepared using conventional methods of differential centrifugation as described previously [28]. Briefly, the animals were anaesthetized as above and the organs were immediately excised and finely minced in an ice-cold isolation medium [10 mM Hepes/KOH, pH 7.4, 250 mM sucrose, 0.5 mM EGTA and 0.1 % defatted BSA (Sigma–Aldrich)]. The minced blood-free heart tissue was then resuspended in 40 ml of isolation medium containing 1 mg of the protease Subtilopeptidase A type VIII (Sigma–Aldrich) per g of tissue and homogenized with a tightly fitting homogenizer (Teflon pestle). The suspension was incubated for 1 min at 4 °C and then re-homogenized. The homogenate was then centrifuged at 14500 g for 10 min. The supernatant fluid was decanted and the pellet, which was essentially devoid of protease, was gently re-suspended in its original volume (40 ml) with a loose-fitting homogenizer. The suspension was centrifuged at 750 g for 10 min and the resulting supernatant was centrifuged at 12000 g for 10 min. The pellet was resuspended using a paintbrush and re-pelletted at 12000 g for 10 min.

The blood free liver tissue was also resuspended in 40 ml of isolation medium and mechanically homogenized. The homogenate was then centrifuged at 800 g for 10 min and the resulting supernatant was centrifuged at 10000 g for 10 min. The mitochondrial pellet was resuspended using a paintbrush and centrifuged twice at 10000 g for 10 min to obtain a final mitochondrial suspension.

For both isolation procedures, EGTA and defatted BSA were omitted from the final washing medium. Mitochondrial and homogenate protein contents were determined by the Biuret method calibrated with BSA [29]. All isolation procedures were performed at 0–4 °C. Considering the relatively greater abundance of IMF (intermyofibrillar) (∼80 %) compared with SS (subsarcolemmal) (∼20 %) mitochondria within the cells, a potentially dominant role for the IMF subtraction compared with the SS subtraction is expected when studying treatment-based mitochondrial alterations.

An aliquot of heart and liver mitochondrial suspension was taken after isolation and prepared for later semiquantification of proteins by Western blotting as detailed below. The remaining fresh heart and liver mitochondrial suspensions were used within 4 h for in vitro assays of mitochondrial oxygen consumption and transmembrane potential and were maintained on ice (0–4 °C) throughout this period. Isolation procedures yielded well-coupled mitochondria: the RCR (respiratory control ratio) of isolated mitochondria varied from 5–8 (with glutamate/malate) or 3–4 (with succinate plus rotenone) for controls, as determined according to the method of Estabrook [30].
Mitochondrial oxygen consumption assays

Mitochondrial respiratory function was measured polarographically, at 25 °C, using a Biological Oxygen Monitor System (Hansatech Instruments) and a Clark-type oxygen electrode (Hansatech DW 1). Reactions were conducted in a 0.75 ml closed thermostatted and magnetically stirred glass chamber containing 0.5 mg of mitochondrial protein in respiration buffer (65 mM KCl, 125 mM sucrose, 10 mM Tris, 20 µM EGTA and 2.5 mM KH2PO4, pH 7.4). After 1 min of equilibration, mitochondrial respiration was initiated by adding glutamate/malate to a final concentration of 10 and 5 mM respectively, or 10 mM succinate plus 4 µM rotenone. State 3 respiration was determined after adding 200 µM ADP; state 4 was measured as the rate of oxygen consumption in the absence of ADP. The RCR (state 3/state 4) and the ADP/O ratio (nmols ADP phosphorylated/nmols O2 consumed) were calculated according to Estabrook [30], using 474 ng of oxygen atoms/ml as the value for oxygen solubility at 25 °C in doubly distilled water.

$\Delta \Psi$ (mitochondrial membrane potential)

$\Delta \Psi$ was monitored indirectly based on the activity of the lipophilic cation TPP+ (tetraphenylphosphonium), as followed with a TPP+ -selective electrode prepared in our laboratory as described by Kamo et al. [31] using a silver chloride reference electrode (Tacussel; Model MI 402). Both the TPP+ electrode and the reference electrode were inserted into an open vessel with magnetic stirring and connected to a pH meter. The signals were fed into a potentiometric recorder. No correction factor was used to correct for the passive binding contribution of TPP+ to membrane potential as the purpose of the present study was to show relative changes in the potential rather than absolute values. As a consequence, a slight overestimation of the $\Delta \Psi$ values is anticipated. The $\Delta \Psi$ was estimated from the following equation (at 25 °C): $\Delta \Psi = 59 \times \log (v/V) - 59 \times \log (10 \Delta E/59 - 1)$, where $v$, $V$, and $\Delta E$ stand for mitochondrial volume, voltage of the incubation medium, and deflection of the electrode potential from the baseline respectively.

A mitochondrial matrix volume of 1.1 µl/mg of protein was assumed. Reactions were carried out in 1 ml of respiration buffer, supplemented with 3 µM TPP+ and 0.5 mg/ml of protein, with the temperature maintained at 25 °C. For measurements of $\Delta \Psi$ with complex I substrates, energization was carried out with 10 mM of glutamate and 5 mM of malate, and ADP-induced phosphorylation was achieved by adding 200 µM ADP. For measurements of $\Delta \Psi$ with complex II substrates, 10 mM succinate plus 4 µM rotenone was added to the medium containing 3 µM TPP+ and mitochondria. The lag-phase, which reflects the time needed to phosphorylate the added ADP, was also measured for both substrates.

Determination of mitochondrial calcium accumulation and MPTP induction

Mitochondrial calcium accumulation capacity was determined by adding small pulses of calcium (100 and 20 nmols of each, for heart and liver mitochondria respectively, until MPTP opening was observed as an irreversible fall in $\Delta \Psi$). The reaction mixture was continuously stirred and the temperature was maintained at 25 °C. The assays were performed in 1 ml of reaction medium (200 mM sucrose, 10 mM Tris, 10 µM EGTA and 5 mM KH2PO4, pH 7.4) supplemented with 4 µM rotenone, 10 mM succinate with 0.5 mg of protein/ml. Control trials were made by using 1 µM of cyclosporine-A, the selective MPTP inhibitor [32].

Levels of cTnl, GOT and GTP

cTnl concentration was quantitatively determined with an established immunoassay using a commercially available Abbott kit (Amadora). Plasma activities of GOT and GTP were determined spectrophotometrically using a commercially available ABX Diagnostics kits.

Respiratory complex activities

Mitochondrial membranes were disrupted by freeze–thaw cycles to allow free access to substrates. All the assays were performed at 30 °C in a final volume of 500 µl. Complex I activity was measured by following the reduction of DCIP (2,6-dichlorophenolindophenol) at 600 nm for 4 min, after which 1 µM (final concentration) rotenone was added and the absorbance was measured again for 4 min [33]. Complex II activity was determined as described previously by Birch-Machin et al. [34]. Briefly, the catalysed reduction of DCIP by the protein complex was followed at 600 nm for 3 min, after addition of 65 µM ubiquinone. Complex V ATP synthase activity was measured according to the method of Simon et al. [35]. Phosphate produced by hydrolysis of ATP reacts with ammonium molybdate in the presence of reducing agents to form a blue-colour complex, the intensity of which is proportional to the concentration of phosphate in solution. Oligomycin was used as an inhibitor of mitochondrial ATPase activity.

Measurement of ANT (adenine nucleotide translocase), VDAC (voltage-dependent anion channel), CypD (cyclophilin D), Bcl-2 and Bax protein levels

Equivalent amounts of proteins obtained from heart and liver mitochondrial suspensions were subjected to SDS/PAGE (15 % gels) as described by Laemmli [36], followed by blotting on to a nitrocellulose membrane (Hybond; Amersham Pharmacia Biotech) according to the method of Locke et al. [37]. Membranes were stained with Ponceau S to verify quality of transfer and equal protein loading. After blotting, non-specific binding was
Table 1  Animal data and yield of mitochondrial protein isolation

Values are means ± S.E.M. * P < 0.05 compared with the NE + SAL group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NE + SAL</th>
<th>NE + DOX</th>
<th>EX + SAL</th>
<th>EX + DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight at injection (g)</td>
<td>241.0 ± 24.4</td>
<td>261.5 ± 29.5</td>
<td>277.8 ± 12.3</td>
<td>278.8 ± 9.4</td>
</tr>
<tr>
<td>Body weight at killing (g)</td>
<td>268.3 ± 25.7</td>
<td>233.8 ± 27.9</td>
<td>293.3 ± 12.1</td>
<td>243.5 ± 7.3</td>
</tr>
<tr>
<td>Change in body weight (g)</td>
<td>27.3 ± 3.5</td>
<td>−27.7 ± 1.7∗</td>
<td>15.5 ± 1.1∗</td>
<td>−34.5 ± 2.9∗</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>0.78 ± 0.04</td>
<td>0.53 ± 0.06∗</td>
<td>0.08 ± 0.01</td>
<td>0.64 ± 0.03</td>
</tr>
<tr>
<td>Heart weight/body weight (mg of tissue/g of body weight)</td>
<td>2.99 ± 0.15</td>
<td>2.31 ± 0.08∗</td>
<td>2.94 ± 0.09</td>
<td>2.65 ± 0.16∗</td>
</tr>
<tr>
<td>Plasma cTnI (ng of protein/ml)</td>
<td>0.00 ± 0.00</td>
<td>1.20 ± 0.31∗</td>
<td>0.14 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Yield of heart mitochondria (mg of mitochondrial protein/g of tissue)</td>
<td>20.7 ± 2.3</td>
<td>22.8 ± 6.4</td>
<td>18.6 ± 2.1</td>
<td>23.5 ± 1.9</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>9.6 ± 1.0</td>
<td>7.3 ± 0.7</td>
<td>9.4 ± 0.6</td>
<td>7.6 ± 0.2</td>
</tr>
<tr>
<td>Liver weight/body weight (mg of tissue/g of body weight)</td>
<td>3.6 ± 0.8</td>
<td>3.2 ± 1.5</td>
<td>3.2 ± 0.9</td>
<td>3.2 ± 1.8</td>
</tr>
<tr>
<td>Plasma GOT (units/l)</td>
<td>162.5 ± 13.3</td>
<td>244.5 ± 25.8∗</td>
<td>166.1 ± 19.0</td>
<td>230.1 ± 17.6∗</td>
</tr>
<tr>
<td>Plasma GPT (units/l)</td>
<td>49.3 ± 7.4</td>
<td>44.3 ± 3.5</td>
<td>54.2 ± 4.6</td>
<td>50.7 ± 2.9</td>
</tr>
<tr>
<td>Yield of liver mitochondria (mg of protein/g of body weight)</td>
<td>6.51 ± 0.8</td>
<td>9.7 ± 0.4</td>
<td>5.8 ± 0.4</td>
<td>6.7 ± 0.5</td>
</tr>
</tbody>
</table>

Mn-SOD (superoxide dismutase) activity

Heart and liver Mn-SOD activity was determined spectrophotometrically according to the method of Marklund and Marklund [38] by monitoring the rate of autoxidation of pyrogallol at 420 nm. The reaction buffer (25 °C) consisted of 1 mM DTPA {N,N-bis[2-[bis(carboxymethyl)amino]-ethyl} glycine] and 50 mM Tris/HCl, pH 8.2, in a 1 ml final volume. Pyrogallol (0.3 mM) was added to the cuvette to start the reaction and the rate of absorbance increase was measured. One unit of SOD activity was defined as the amount of sample required to inhibit the autoxidation of pyrogallol by 50%, and the activity is expressed as units/mg of protein.

Statistical analysis

Means and S.E.M. were calculated for all variables in each group. Two-way ANOVA followed by the Bonferroni post-hoc test was used to compare groups. The Statistical Package for the Social Sciences (SPSS, version 10.0) was used for all analyses. The significance level was set at 5%.

RESULTS

As can be seen from Table 1, there were no differences in the mean body weight of the animals among the experimental groups at the time of injection. Significant decreases were observed in body weight at 5 days after the DOX treatment, although not independently of the exercise status, as the body weight in the EX + DOX group was also lower than in the NE + SAL group. Absolute heart weight, as well as the heart to body weight ratio, in the NE + DOX and EX + DOX groups was significantly lower than in the NE + SAL group. Significant increases...
in the plasma content of cTnI were observed in the NE + DOX and EX + DOX groups when compared with the NE + SAL group (Table 1). Plasma levels of GOT and GTP were determined as combined markers of liver damage. DOX treatment induced an increase in GOT (NE + SAL group compared with the NE + DOX and EX + DOX group).

Considering the high acute bolus of DOX administrated to the animals and the 5 days between the injection and killing, some level of mortality was expected. However, the percentage of rat survival was 100%, as no animal had died during this period.

### Oxygen consumption and ΔΨ

#### Heart mitochondria

To elucidate whether a single acute running bout performed 24 h before DOX treatment could prevent the DOX-associated cardiac mitochondrial dysfunction, oxygen consumption (Table 2) and ΔΨ (Table 3) were determined with substrates for both complex I and II.

Using complex I and II substrates, DOX treatment caused a significant decrease in heart mitochondrial state 3 respiratory rates in the non-exercised groups (NE + SAL group compared with the NE + DOX group), which was prevented by exercise. Acute running exercise in itself (NE + SAL group compared with the EX + SAL group) increased the RCR when glutamate/malate was used as substrate. When compared with NE + DOX group, an increased state 4 respiratory rate was observed in the EX + DOX group compared with the NE + DOX group when succinate was used. As seen in Table 3, DOX treatment caused a significant decrease in the maximal ΔΨ developed and in the repolarization after ADP phosphorylation in the non-exercised groups (NE + SAL group compared with the NE + DOX group). The decrease in both parameters was counteracted by previous exercise. In addition, an important result is that DOX increased the time required for the phosphorylation of the added ADP, a negative effect which was antagonized by acute exercise.

#### Liver mitochondria

DOX and exercise induced fewer alterations in the functional endpoints evaluated in liver mitochondria during oxygen consumption and ΔΨ experiments when compared with the results in heart mitochondria in which the effects were more evident. Nevertheless, an unexpected increase in state 3 respiration caused by DOX treatment (NE + SAL group compared with the NE + DOX group) was observed when succinate was used as the substrate.

### Calcium accumulation and MPTP induction

#### Heart mitochondria

Calcium-induced ΔΨ depolarization is a typical phenomenon that is associated with MPTP induction. Figure 1 and Table 4 show the alterations of heart mitochondrial electric potential induced by DOX and exercise during the addition of several calcium pulses until the threshold for MPTP opening was reached.

Regarding heart mitochondria, DOX treatment induced a decrease in both the ΔΨ developed after the addition of five calcium pulses and in the total number of calcium pulses before MPTP opening, when compared to all other groups. Therefore, heart mitochondria from exercised rats were able to accumulate more calcium than their non-exercised counterparts, when later treated with DOX (NE + DOX group compared with the EX + DOX group).

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**Table 2** Respiratory parameters in heart mitochondria isolated from each experimental group

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment group</th>
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<tbody>
<tr>
<td></td>
<td>NE + SAL</td>
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</table>

| Glutamate/malate substrate | | |
|---------------------------|--|---|---|---|
| State 3 rate (ng of atoms of oxygen · min⁻¹ · mg⁻¹ of protein) | 414.0 ± 18.5 | 334.0 ± 20.7* | 404.4 ± 10.3 | 401.6 ± 22.9 |
| State 4 rate (ng of atoms of oxygen · min⁻¹ · mg⁻¹ of protein) | 813.3 ± 9.5 | 72.1 ± 6.5 | 64.3 ± 12.6 | 88.1 ± 10.9 |
| RCR | 5.9 ± 0.1 | 4.7 ± 0.23 | 7.8 ± 0.8# | 5.4 ± 0.1 |

| Succinate substrate | | |
|---------------------|--|---|---|---|
| State 3 rate (ng of atoms of oxygen · min⁻¹ · mg⁻¹ of protein) | 490.2 ± 34.2 | 383.0 ± 12.3# | 508.0 ± 32.2 | 494.8 ± 12.9 |
| State 4 rate (ng of atoms of oxygen · min⁻¹ · mg⁻¹ of protein) | 218.5 ± 17.4 | 158.0 ± 14.9 | 243.2 ± 19.4 | 261.6 ± 13.5† |
| RCR | 2.6 ± 0.1 | 2.0 ± 0.9 | 3.4 ± 0.4 | 2.4 ± 0.1 |

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Table 3  \( \Delta \Psi \) fluctuations (maximal energization, ADP-induced depolarization, and repolarization) and ADP phosphorylation lag phase of glutamate/malate- and succinate-energized heart mitochondria isolated from each experimental group

Results are means \( \pm \) S.E.M. for heart mitochondria (1 mg of protein/ml) obtained from different mitochondrial preparations for each experimental group. The Table shows the average response of \( \Delta \Psi \) developed with 10 mM glutamate/5 mM malate, or 10 mM succinate (energization), the decrease in membrane potential after ADP addition (depolarization), and the repolarization value after ADP phosphorylation. \( \Delta \Psi \) was measured using a TPP\(^+\)-selective electrode at 25 \( \degree \)C in a total volume of 1 ml. \#\( P < 0.05 \) compared with all other groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NE + SAL</th>
<th>NE + DOX</th>
<th>EX + SAL</th>
<th>EX + DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate/malate</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Maximal energization ( \Delta \Psi ) (( \text{mV} ))</td>
<td>222.4 ( \pm ) 1.9</td>
<td>214.2 ( \pm ) 1.4#</td>
<td>224.0 ( \pm ) 1.1</td>
<td>223.0 ( \pm ) 1.7</td>
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<tr>
<td>ADP depolarization ( \Delta \Psi ) (( \text{mV} ))</td>
<td>41.5 ( \pm ) 2.5</td>
<td>39.3 ( \pm ) 0.3</td>
<td>43.2 ( \pm ) 4.5</td>
<td>36.6 ( \pm ) 1.2</td>
</tr>
<tr>
<td>Repolarization ( \Delta \Psi ) (( \text{mV} ))</td>
<td>219.6 ( \pm ) 2.0</td>
<td>210.5 ( \pm ) 1.5#</td>
<td>221.6 ( \pm ) 1.2</td>
<td>223.0 ( \pm ) 1.9</td>
</tr>
<tr>
<td>Lag-phase (s)</td>
<td>32.8 ( \pm ) 2.7</td>
<td>58.2 ( \pm ) 4.5#</td>
<td>29.3 ( \pm ) 1.1</td>
<td>29.3 ( \pm ) 3.6</td>
</tr>
<tr>
<td>Succinate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximal energization ( \Delta \Psi ) (( \text{mV} ))</td>
<td>226.2 ( \pm ) 1.0</td>
<td>217.7 ( \pm ) 2.6#</td>
<td>230.9 ( \pm ) 0.1</td>
<td>227.1 ( \pm ) 2.4</td>
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<tr>
<td>ADP depolarization ( \Delta \Psi ) (( \text{mV} ))</td>
<td>66.8 ( \pm ) 4.6</td>
<td>53.2 ( \pm ) 2.6</td>
<td>61.4 ( \pm ) 3.3</td>
<td>48.7 ( \pm ) 1.5</td>
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<tr>
<td>Repolarization ( \Delta \Psi ) (( \text{mV} ))</td>
<td>225.6 ( \pm ) 0.9</td>
<td>217.5 ( \pm ) 2.5#</td>
<td>230.6 ( \pm ) 0.1</td>
<td>228.4 ( \pm ) 2.1</td>
</tr>
<tr>
<td>Lag-phase (s)</td>
<td>46.8 ( \pm ) 2.8</td>
<td>64.9 ( \pm ) 1.9#</td>
<td>39.0 ( \pm ) 1.9</td>
<td>44.3 ( \pm ) 3.6</td>
</tr>
</tbody>
</table>

Figure 1  Calcium accumulation capacity of heart mitochondria freshly isolated from each animal

Calcium accumulation capacity, measured with a TPP\(^+\)-selective electrode at 25 \( \degree \)C in a total volume of 1 ml, was assessed by adding several pulses of CaCl\(_2\) (each of 100 nmol/mg of protein) in order to induce the MPTP. The opening of MPTP was determined as a fall in \( \Delta \Psi \). In the presence of cyclosporin A, a specific inhibitor of MPTP induction, the number of pulses of calcium needed to induce a similar decrease in membrane potential is much higher for all mitochondrial preparations from each group (results not shown). The traces are representative of experiments performed with different mitochondrial preparations from the indicated treatment groups.

Liver mitochondria

Exercise in itself (NE+ SAL group compared with the EX+ SAL group) increased the capacity of liver mitochondria to maintain \( \Delta \Psi \) after three calcium pulses, although no differences between groups were found regarding the total number of calcium pulses added before MPTP opening (Table 4).

Respiratory complex activities

Heart mitochondria

The activities of respiratory complexes I, II and V were also determined in heart and liver mitochondria (Table 5). As shown in Table 5, acute exercise prevented the impairment of heart mitochondrial complex I activity and attenuated the decreased complex V activity caused by DOX. Interestingly, exercise in itself (NE+ SAL group compared with the EX+ SAL group) increased heart mitochondrial ATP synthase (complex V) activity.

Liver mitochondria

Although no significant effects of DOX were observed in the activities of the analysed complexes in the livers of non-exercised rats (NE+ SAL group compared with the NE+ DOX group), the combination treatment (EX+ DOX) resulted in an increased activity of the complexes compared with the NE+ DOX group. Exercise alone also increased liver mitochondrial complex I, II and V activities.

Apoptotic signalling and MPTP-related proteins

Heart and liver

As markers for apoptotic signalling, we measured heart tissue caspase 3 and 9 activities. As can be observed in
Table 4  Calcium accumulation capacities of freshly isolated heart and liver mitochondria energized with 10 mM succinate obtained from each group

Results are means ± S.E.M. for heart (1 mg of protein/ml) and liver (0.5 mg of protein/ml) mitochondria. The Table shows the average response of $\Delta$Ψ developed after five calcium pulses of 100 nmol (heart) and after three pulses of 20 nmol (liver), and the number of calcium pulses before the MPTP-induced $\Delta$Ψ fall. $\Delta$Ψ was measured using a TPP$^+$-selective electrode at 25°C in a total volume of 1 ml. *P < 0.05 compared with the NE + SAL group; #P < 0.05 compared with all other experimental groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NE + SAL</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
</tr>
<tr>
<td>$\Delta$Ψ after 5 pulses of 100 nmol calcium (−mV)</td>
<td>214.3 ± 6.8</td>
</tr>
<tr>
<td>Calcium pulses (number)</td>
<td>9.3 ± 1.7</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td>$\Delta$Ψ after 3 pulses of 20 nmol calcium (−mV)</td>
<td>196.1 ± 7.8</td>
</tr>
<tr>
<td>Calcium pulses (number)</td>
<td>3.3 ± 0.4</td>
</tr>
</tbody>
</table>

Table 5  Heart and liver mitochondrial chain complex activities

Results are means ± S.E.M. *P < 0.05 compared with the NE + SAL group; #P < 0.05 compared with all other groups; †P < 0.05 compared with the NE + DOX group.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NE + SAL</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
</tr>
<tr>
<td>Complex I (units/g of protein)</td>
<td>1.17 ± 0.08</td>
</tr>
<tr>
<td>Complex II ($\mu$mol.min$^{-1}$.g$^{-1}$ of protein)</td>
<td>3.06 ± 0.26</td>
</tr>
<tr>
<td>Complex V ($\mu$mol P$\text{I}$.min$^{-1}$.mg$^{-1}$ of protein)</td>
<td>3.39 ± 0.06</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td>Complex I (units/g of protein)</td>
<td>0.54 ± 0.10</td>
</tr>
<tr>
<td>Complex II ($\mu$mol.min$^{-1}$.g$^{-1}$ of protein)</td>
<td>1.03 ± 0.08</td>
</tr>
<tr>
<td>Complex V ($\mu$mol P$\text{I}$.min$^{-1}$.mg$^{-1}$ of protein)</td>
<td>0.78 ± 0.07</td>
</tr>
</tbody>
</table>

Immunoblotting was then performed in order to semiquantify heart and liver mitochondrial proteins that are known to be involved in mitochondrial apoptotic signalling, including the pro and anti-apoptotic Bcl-2 family proteins, Bax and Bcl-2, respectively as well as MPTP component and/or sensitizing proteins, such as ANT, VDAC and CypD (Figures 3 and 4). However, none of the stimuli either isolated or combined caused significant changes in any of these proteins.

Mn-SOD activity

Heart and liver

In order to measure an end-point for mitochondrial antioxidant enzymatic protection, the activity of the mitochondrial specific isoform of SOD in liver and heart was determined (Figure 5). Both DOX (NE + SAL group compared with the NE + DOX group) and exercise (NE + SAL group compared with the EX + SAL group) in itself induced an increase of SOD activity in heart mitochondria. In liver mitochondria, the only observed increase in SOD activity was induced by exercise alone.

Figure 2  Effect of DOX treatment and acute exercise on caspase 3 and caspase 9 levels of heart tissue

Results are means ± S.E.M. #P = 0.05 compared with all the other groups.

Figure 2, DOX increased both caspase 3 and 9 activities in non-exercised (NE + SAL group compared with the NE + DOX group) animals, an effect which was prevented by the previous single bout of running.
DISCUSSION

In the present work, we tested the hypothesis that impairment in mitochondrial bioenergetics, including increased susceptibility to calcium-induced MPTP opening in isolated heart mitochondria, after acute DOX treatment are prevented by 60 min of treadmill running performed 24 h prior to DOX treatment. Our hypothesis was confirmed as mitochondrial functions, including calcium accumulation capacity, that were affected by DOX were improved by exercise. However, no large body of conclusive results were obtained regarding the mechanisms associated with decreased apoptotic signalling provided by exercise in DOX-treated rats, as seen by the results on caspase 3 and 9 activities. In fact, MPTP regulatory and/or structural components, such as Bax, Bcl-2, ANT, CypD and VDAC, were unchanged in all experimental groups.

Figure 3  Semi-quantification of heart and liver mitochondrial Bax (upper panel) and Bcl-2 (lower panel) protein expression
Results are means ± S.E.M. for each experimental condition. Typical immunoblots of Bax and Bcl-2 are presented below the histograms.

Figure 4  Semi-quantification of heart and liver mitochondrial ANT (top panel), CypD (middle panel) and VDAC (bottom panel) protein expression
Results are means ± S.E.M. for each experimental condition. Typical immunoblots of VDAC, CypD and ANT are presented below the histograms.
The results on mitochondrial function obtained in the present study confirm, at least in part, the cardiac protection afforded by acute exercise previously described against deleterious stress stimuli [21–24], particularly acute single-dose DOX treatment [25].

Cardiomyocytes incubated with DOX [40]. observed in the present study agrees with the impairment of mitochondrial function in DOX-treated animals has been studied previously [2,3]. The present study confirmed that DOX administration results in decreased heart mitochondrial respiration, maximally developed $\Delta\Psi$ and an increased phosphorylative lag phase (Tables 2 and 3). Moreover, DOX decreased the ability of heart mitochondria from non-exercised rats to accumulate calcium before MPTP opening (Table 4 and Figure 1). The acute bout of running performed 24 h before DOX treatment resulted in attenuation or complete prevention of the heart mitochondrial impairments induced by DOX.

Alterations in mitochondrial oxidative phosphorylation induced by DOX could have their origin in several factors: (i) inactivation of dehydrogenases, providing limited amounts of reducing equivalents to the mitochondrial electron transport chain; (ii) decreased aconitase activity; and (iii) decreased activity, content or organization of electron transport chain complexes or proteins of the phosphorylation system.

Sequestration of excess cytosolic calcium by mitochondria represents an important cytoprotective mechanism in the latter stages of cell injury [39]. The interplay between mitochondrial fidelity and cell calcium homoeostasis is exemplified by the fact that calcium enters the mitochondrial matrix via an electrophoretic mechanism [39]. Consequently, the inward flux of calcium represents a depolarizing current, the strength of which is determined by the $\Delta\Psi$, which itself is a function of the fidelity of the mitochondrial electron transport chain. The significant decrease in mitochondrial $\Delta\Psi$ observed in the present study agrees with the impairment reported in cardiomyocytes incubated with DOX [40]. It is however important to note that the $\Delta\Psi$ values above $-200$ mV in all experimental groups do not seem to compromise ATP synthase flow or the transport of ions and metabolites. In fact, the range of the $\Delta\Psi$ is $-120$ to $-220$ mV. For instance, regarding the driving force for ATP generation, it has been shown that the kinetics of the ATP synthase follow a sigmoid pattern in response to $\Delta\Psi$, reaching saturation at approximately $-100$ mV [41].

The effect of exercise may be associated with the preservation of mitochondrial complex activity, namely complex I and V, in the EX + DOX group in opposition to the decreased activity observed in the NE + DOX group (Table 5). In fact, mitochondrial complex I of the respiratory chain catalyses the futile redox cycling of DOX with consequent generation of free radicals [42,43], which is the activity of this complex affected in the process [4]. Previous studies have also reported DOX-induced impairments in mitochondrial ATPase activity or in the redox state of respiratory carriers [44]. As opposed to the increment in heart mitochondrial complex V observed in the present study, in which animals were killed at 6 days after 60 min of endurance exercise, Bo et al. [45] described no alterations in heart mitochondrial ATP synthase immediately after acute exercise. Possible differences in the time between the end of exercise and mitochondrial sampling may be the cause of these discrepancies.

As mentioned, one early and sensitive marker of DOX toxicity is related to the increased susceptibility to MPTP opening, although not exclusively in heart mitochondria. Mitochondria isolated from sedentary animals treated for 7 weeks with cumulative DOX doses revealed a decreased ability to accumulate calcium caused by enhanced MPTP induction [4–6,9]. In addition, liver mitochondria from DOX-treated rats also show increased sensitivity to MPTP induction [4,46]. Several important pharmacological and non-pharmacological countermeasures have been successfully used against DOX-induced mitochondrial toxicity, including antioxidants and physical exercise [5,11]. In fact, physical exercise, particularly endurance training, has proven to positively modulate heart MPTP dynamics [47–49].

The results from the present study provide for the first time evidence that 60 min of endurance treadmill running afforded protection against the increased sensitivity of the heart to MPTP opening caused by DOX administration (Table 4 and Figure 1). The calcium accumulation capacity of heart and liver mitochondria from each of the four experimental groups was determined by following the effect of known amounts of calcium (pulses of 100 and 20 nmol of CaCl$_2$ each for heart and liver mitochondria respectively) on mitochondrial $\Delta\Psi$. Mitochondria possess a finite capacity for accumulating calcium before undergoing calcium-dependent MPTP opening in a phosphate-rich medium, resulting in irreversible apoptosis. Our results showed that heart mitochondria...
from the NE + DOX group of rats have lower calcium tolerance than all other groups, including the EX + DOX group (Figure 1). These results show that acute exercise activated cellular and/or mitochondrial defence mechanisms that probably contributed to prevent the increased MPTP opening susceptibility caused by DOX. Regarding liver mitochondria, no cross tolerance effect was observed as DOX did not alter calcium accumulation capacity and only the EX + SAL group demonstrated a higher $\Delta \Psi$ developed after three pulses of 20 nmol of calcium when compared with the NE + SAL group.

In an attempt to better understand the mechanisms that might contribute to the observed functional phenotypes (see above), we quantified the levels of several potential proteins known to be involved in the modulation of the pore, either as components and/or sensitizers, including ANT, CypD, VDAC, Bax and Bcl-2 (Figures 3 and 4).

Calcium-induced MPTP opening is modulated by a variety of physiological effectors. It is increasingly recognized that the molecular composition of the pore is likely to be variable [50,51]; despite this, the prevailing hypothesis is that ANT, VDAC and the regulatory matrix CypD proteins are the most common components forming the MPTP complex [52,53]. In fact, despite some conflicting results [54,55], variations in the level of expression of the MPTP component/regulatory proteins ANT and CypD were reported to correlate with the susceptibility of isolated mitochondria to undergo MPTP opening and decisively contribute to mitochondrial dysfunction [56,57]. In addition, it was shown previously that the toxicity caused by DOX in cardiac tissue involved a decrease in ANT levels [9]. However, this was not observed in the present study as none of these important proteins was significantly altered by DOX and/or exercise (Figure 3). It is possible that the functional results obtained are related to the co-ordinated modulation of other mechanisms involving for instance stress chaperones, antioxidants or other defence systems. In fact, it is known that in response to endurance exercise cardiac tissue up-regulates the levels of heat-shock proteins [58], which act as molecular chaperones, possibly contributing to the preservation of the integrity and activity of mitochondrial complexes. The protective role of heat-shock proteins may be accomplished through facilitation of nuclear-encoded protein importation and assembly in the mitochondrial matrix, and through the improved assisted folding of proteins within mitochondria. Moreover, acute endurance exercise also induces an increase in the content of cardiac uncoupling proteins [45], which are suggested to contribute to a decrease in free radical production by causing a slight dissipation of $\Delta \Psi$ (mild uncoupling) [59] and translocation of fatty acid peroxides from the inner to the outer membrane leaflet [60].

Interestingly, the levels of Mn-SOD activity of heart mitochondria from the NE + DOX and EX + SAL groups and of liver mitochondria from the EX + SAL group were increased when compared with the NE + SAL group, which can be interpreted as a sign of adaptation of both stimuli to the increased oxidative stress imposed. It was intriguing that, although Mn-SOD activity from the NE + DOX and EX + SAL groups had increased, no up-regulation in activity was detected when exercise and DOX were combined (EX + DOX group). One possible explanation is that both stimuli combined may lead to up-regulation of other antioxidant defence mechanisms, which would make increased expression of Mn-SOD unnecessary to counteract any particular oxidative stress. Further work in this area is warranted.

Mitochondrial (dys)function is increasingly considered a key event in a variety of forms of cell death, including apoptosis. Therefore we also analysed the effects of DOX and exercise on caspase-3 and -9 activities (Figure 2) and, as mentioned, on the expression of the pro- and anti-apoptotic Bcl-2 family proteins Bax and Bcl-2 (Figure 3). The relative expression of the later in the mitochondrial outer membrane is thought to mediate the fate of the cell by regulating outer membrane integrity and the consequent release of mitochondrial pro-apoptosis proteins, such as cytochrome $c$ or the apoptosis-inducing factor [61]. Moreover, results suggest that Bax, and perhaps its homologues Bak and Bid, may regulate MPTP opening considering that these effects are antagonized by anti-apoptotic Bcl-2 family members [50]. Unexpectedly, our results do not confirm previous findings reporting increased Bax/Bcl-2 at 4–5 days after acute DOX treatment [15,62]. However, the observed increased activities of heart tissue caspase 3 and 9 with DOX in the non-exercised group was prevented by exercise, which suggests that DOX-induced in vivo increased downstream cellular and mitochondrial-related apoptotic events that were prevented by exercise.

In conclusion, our results indicate that the respiratory dysfunction and the increased susceptibility to MPTP gating of heart mitochondria isolated from DOX-treated rats can be prevented by an acute bout of endurance treadmill running. These mitochondrial adaptations could potentially be beneficial to the cardiac tissue in the setting of DOX cardiomyopathy, a condition in which acute exercise was shown to be protective [25]. Considering that DOX can result in a mitochondrial disease with consequent loss of organelle and tissue functions, non-pharmacological countermeasures, such as physical exercise, that can afford protection against this mitochondrialopathy should be taken into account, due to their positive effects.

**AUTHOR CONTRIBUTION**

António Ascensão, José Lumini-Oliveira, Nuno Machado, Rita Ferreira, Inês O. Gonçalves, Ana Moreira, Franklin Marques, Vilma Sardão, Paulo Oliveira and José
Magalhães were significant contributors, providing the concept, as well as the experimental design of the study and writing of the manuscript. All authors performed the functional and biochemical experiments and gave various contributions in the interpretation of the results and in the critical revision of the manuscript draft.

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