Doxorubicin-induced cardiac dysfunction is attenuated by ciclosporin treatment in mice through improvements in mitochondrial bioenergetics

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We tested whether inhibition of mitochondrial membrane potential dissipation by CsA (ciclosporin A) would prevent doxorubicin-induced myocardial and mitochondrial dysfunction. Acute and subchronic models of doxorubicin exposition were performed in mice with either a single intraperitoneal bolus (10 mg/kg of body weight, intraperitoneal) or one injection of 4 mg · kg⁻¹ of body weight · week⁻¹ during 5 weeks. Follow-up was at 1.5 weeks and 16 weeks in acute and subchronic models respectively. Mice received either CsA (1 mg/kg of body weight, intraperitoneal on alternate days) or saline until follow-up. Heart function was evaluated by echocardiography. Mitochondrial measurements included oxygen consumption, membrane potential and externally added calcium-induced mitochondrial permeability transition. Mitochondrial mass was evaluated by transmission electronic microscopy and mtDNA (mitochondrial DNA) content. Mitochondrial dynamics were detected as the expression of GTPases involved in mitochondrial fusion and fission. In both the acute and chronic models, doxorubicin exposure was associated with increased mtDNA content, mitochondrial fragmentation and changes in mitochondrial fusion- and fission-related transcripts [increases in Mfn2 (mitofusin 2), Opal (optic atrophy 1 homologue) and Fisl (fission 1 homologue), and no changes in Drp1 (dynamin 1-like)]. CsA did not alter mitochondrial biogenesis, but prevented mitochondrial fragmentation and partially restored the mitochondrial energy-producing capacity. These findings suggest that in vivo CsA treatment may limit MPTP (mitochondrial permeability transition pore) opening, mitochondrial potential loss and contractile depression in acute and chronic models of cardiac toxicity induced by doxorubicin.

Key words: biogenesis, ciclosporin, doxorubicin, energetics, heart, mitochondrial transition permeability.

Abbreviations: Actb, β-actin; CsA, ciclosporin A; Drp1, dynamin 1-like; Fis1, fission 1 homologue; LDH, lactate dehydrogenase; LVFS, left ventricular fraction shortening; Mfn2, mitofusin 2; MPTP, mitochondrial permeability transition pore; mtDNA, mitochondrial DNA; Opal, optic atrophy 1 homologue; PGC-1α (Ppargc1a), peroxisome-proliferator-activated receptor γ co-activator-1α; RCR, respiratory control ratio; ROS, reactive oxygen species; TPP, tetraphenylphosphonium.

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INTRODUCTION

Doxorubicin is an anthracycline antibiotic that is widely used as a chemotherapeutic agent for haematological malignancies and solid tumours, yet its usefulness is limited by cardiac toxicity [1]. Administration of doxorubicin induces numerous acute cardiac toxic effects, including transient arrhythmias, non-specific electrocardiographic abnormalities, pericarditis and transient depression of left ventricular function [1,2]. In addition, high cumulative doses are associated with late-onset cardiomyopathy that is refractory to standard treatment. For example, doxorubicin has been reported to induce heart failure in up to 30% patients receiving 500 mg/m² or higher cumulative dose with poor clinical outcome [1,2]. Both of the acute and chronic forms of anthracycline-induced cardiac toxicity have been related to mitochondrial production of ROS (reactive oxygen species) and mitochondrial calcium overload [3–7]. Studies suggest that unregulated mitochondrial biogenesis [8,9] and rapid fragmentation of the mitochondrial network [10] are also responsible for the pathogenesis of doxorubicin-induced cardiomyopathy.

The major mechanisms of heart dysfunction induced by oxidative stress, including ROS production, protein damage and mitochondrial injury, have been explored in this model [11]. To ameliorate the cardiotoxic side effects of anthracyclines, several strategies, including limitation of the cumulative dose of anthracyclines, different anthracycline dosage schedules or different antioxidant agents, have been used, but provide only modest efficacy [11]. As a different hypothesis, doxorubicin-induced oxidative stress may induce mitochondrial permeability transition, resulting in matrix swelling, uncoupling of the respiratory chain and membrane potential collapse [6]. Interestingly, in vitro pre-incubation of mitochondria with CsA (ciclosporin A), which limits mitochondrial permeability transition by binding to matrix cyclophilin D, reverses the diminished calcium loading capacity of mitochondrial isolated from doxorubicin-treated rats [3,7]. In agreement, mice-lacking cyclophilin D, a putative constituent of the MPTP (mitochondrial permeability transition pore), are protected against doxorubicin cardiotoxicity [12]. We have recently shown that a non-immunosuppressive dose of CsA could prevent doxorubicin-induced mitochondrial alterations and myocardial function in both the acute and chronic models of doxorubicin cardiac toxicity performed in young adult (6–8 weeks of age) male C57BL/6 mice. Our secondary aim was to test whether CsA could prevent doxorubicin-induced mitochondrial proliferation and mitochondrial network fragmentation.

MATERIALS AND METHODS

Mouse models of doxorubicin cardiotoxicity

Male C57BL/6 mice (6–8 weeks of age; Charles Rivers Laboratories, Lyon, France) were housed for 6 days before manipulation. All experiments were conducted in accordance with the National and European Institutes of Health guidelines for the use of laboratory animals and were approved by the University of Lille. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health [NIH (National Institutes of Health) Publication No. 85–23, revised 1996]. Acute (protocol A) and subchronic (protocol B) models of doxorubicin exposure were performed in mice with either a single intraperitoneal bolus (10 mg/kg of body weight, intraperitoneal) or one injection of 4 mg·kg⁻¹ of body weight·week⁻¹ over 5 weeks. Follow-up was at 1.5 weeks and 16 weeks in the acute model and in the subchronic model respectively. Control groups received an intraperitoneal injection of 0.9% saline. Control and doxorubicin-treated mice were randomized to receive intraperitoneal injection (on alternate days until the end of the study) of (i) vehicle (1% ethanol in saline); (ii) CsA (1 mg/kg of body weight); or (iii) tacrolimus (FK506, a ciclosporin derivative with no inhibitory effect on mitochondrial transition pore; 0.1 mg/kg of body weight).

Animal use and experimental design

In the acute doxorubicin exposure protocol (1.5-week follow-up), three independent sets of animals were used for survival study, echocardiography and mitochondrial evaluation or ultrastructure and biochemistry studies. In the subchronic doxorubicin exposure protocol (16-week follow-up), two sets of animals were used for either survival or echocardiography study.

Survival studies

In the both acute and subchronic studies, the survival rate was evaluated twice. An investigator blinded to the identity of the mice performed a 1.5-week follow-up in the acute doxorubicin exposure protocol (protocol A), and a 16-week follow-up in the subchronic doxorubicin exposure protocol (protocol B), in two separate sets of experiments using 20 mice per group.
Echocardiography
Mice from all treatment groups were anaesthetized with isoflurane, and transthoracic echocardiography was performed using Acuson Sequoia C512 system with a 14-MHz linear transducer. Echocardiographic measurements were taken in M-mode in triplicate for each mouse. In accordance with the American Society of Echocardiography guidelines, the LVFS (left ventricular fraction shortening) was calculated as LVFS = (LVEDD – LVESD)/LVEDD, where LVEDD is left ventricular diameter at end diastole and LVESD is left ventricular diameter at end systole. All surviving mice were analysed by echocardiography on week 1.5 (protocol A) or week 16 (protocol B) and then killed for mitochondrial study (protocol A).

Heart mitochondrial fraction preparation
Myocardium was excised and placed in isolation buffer A containing 300 mmol/l sucrose, 5 mmol/l Tes and 0.2 mmol/l EGTA, pH 7.2 (4 °C). The tissue was finely minced and homogenized by the use of a Kontes tissue grinder. After centrifugation at 800 g for 5 min, the supernatant was centrifuged at 8800 g for 5 min. The mitochondrial pellet was resuspended in buffer A and centrifuged one more time at 8800 g for 5 min. Protein concentration was determined according to the Bradford method. The purity and integrity of isolated mitochondria were assessed by measuring specific activities of nicotinamide adenine dinucleotide phosphate-cytochrome c oxidase, as an endoplasmic reticulum marker enzyme and cytochrome c oxidase, as an inner membrane marker enzyme.

Mitochondrial transmembrane potential and permeability transition
Isolated mitochondria (1 mg/ml proteins) were suspended in buffer C (250 mmol sucrose, 10 mM Mops, 5 mM glutamate/Tris, 2 mM malate/Tris, 1 mM Pi/Tris and 0.02 mM EGTA/Tris (pH 7.4)) at 25 °C in a multiport measurement chamber equipped with TPP+ (tetrphenylphosphonium)-selective microelectrodes and reference electrodes (WPI). First, mitochondria were gently stirred for 1.5 min in buffer C containing 1.5 μM TPP+ (Sigma). At the end of the pre-incubation period, 10 μmol/l CaCl2 administration was performed every 90 s with a micro-syringe injector connected to a Micro4 pump controller (UMPII and Micro4, WPI). Each 10 μmol/l CaCl2 pulse was detected as a peak of extramitochondrial calcium concentration. The calcium is then very rapidly taken up by the mitochondria, resulting in a return of extramitochondrial calcium concentration to near baseline level. Following sufficient calcium loading, the so-called calcium retention capacity, extramitochondrial calcium concentration abruptly increases indicating a massive release of calcium by mitochondria due to permeability transition pore opening. The amount of externally added calcium necessary to trigger this massive calcium release is used as an indicator of the susceptibility of MPTP opening to calcium overload. This amount is expressed in nmol/mg of protein. To test for the involvement of MPTP opening in the loss of further calcium accumulation, mitochondria were pretreated with 1 μM CsA, an inhibitor of the MPTP.

Mitochondrial transmembrane potential was estimated by calculating the transmembrane distribution of TPP+. The transmembrane potential ΔΨm was calculated as 59log(τ/V) – 59log(10ΔE/59 – 1), where τ is the matrix volume (1.1 μl/mg of mitochondrial protein), V is the volume of the chamber (1 ml) and ΔE is the voltage difference before and after calcium-induced permeability transition expressed in mV.

Mitochondrial respiratory function in cardiac fibres
Respiratory function of the total mitochondrial population was studied in saponin-skinned fibres prepared from myocardium samples. Briefly, after excision, myocardium was rinsed and placed in the relaxing and biopsy preservation solution BIOPS [2.77 mM CaK2/EGTA, 7.2 mM EGTA (free calcium concentration: 0.1 μM), 5.7 mM sodium ATP, 6.6 mM MgCl2·6H2O, 20 mM taurine, 15 mM sodium phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol and 50 mM Mes, pH 7.1], as described previously [14]. Isolated fibres were permeabilized for 30 min with saponin (50 μg/ml) in BIOPS. Then, fibres were washed three times in respiration medium, Mitomed2 (MitoMedium; Oroboros Instruments) (see below). All procedures were performed at 4 °C. Respiration was then measured at 25 °C in a two-chamber respirometer (Oxygraph-2k; Oroboros Instruments). 5–10 mg of fibre bundles were placed in a chamber containing 2 ml of Mitomed2 (0.5 mM EDTA, 5 mM MgCl2·6H2O, 10 mM KH2PO4, 110 mM mannitol, 60 mM KCl and 60 mM Tris/HCl, pH 7.4). The O2 solubility of this medium was taken as 11.3 μM/kPa.

Respiration rates of 5–10 mg of skinned fibres were then measured at 25 °C in a two-chamber respirometer (Oxygraph-2k oxygrapha) containing 2 ml of Mitomed2 with or without substrates. State 4 respiration (without ADP) was first determined with glutamate (5 mM) + malate (2 mM). State 3 respiration was then achieved by adding ADP (2.5 mM) to the preparation. RCR (respiratory control ratio) was determined by calculating the ratio between ADP-stimulated respiration over state 4 respiration (without ADP) with glutamate and malate as the substrate. At the end of the respiratory protocol, the addition of cytochrome c (10 μM) was used to check mitochondrial outer-membrane permeability. Rates of respiration are given in pmol of O2·s−1·mg−1 of wet weight.
Heart ultrastructure

Mouse hearts were examined by morphometric analysis of mitochondrial volume density and fragmentation. One block of tissue was isolated from the left ventricular free wall of each heart. These blocks were fixed with glutaraldehyde, embedded and oriented for cross-sectional views. Thin sections were stained with uranyl acetate and lead citrate, and examined using a Phillips CM10 transmission electron microscope. An individual who was blinded to the treatment groups performed the electron microscopic examination of sections, photographed ten representative prints per block, made prints at standardized magnification ($\times 12300$) and performed point counting using a standard grid overlay. The grid was 18 cm $\times$ 18 cm with lines spaced at 1 cm. Each of the grid intersects was scored by the blinded individual to determine if the intersect fell upon a mitochondrial profile or was outside of mitochondria. The ratio (number of grid intersects superimposing mitochondrial profiles/number of total possible intersects) represents the mitochondrial volume density, which was averaged for each heart. In longitudinally sectioned cardiomyocytes, lengths of individual mitochondria were measured using Metamorph software. For each heart, approx. 1000 mitochondria were measured on ten representative prints ($\times 12300$) to determine the percentage distribution of mitochondria with various lengths: 0–1.5 μm; 1.5–2 μm (sarcomere length); and $\geq $2 μm. An index of mitochondrial fragmentation was the percentage of mitochondria of more than 1.5 μm.

mtDNA (mitochondrial DNA) copy number and real-time PCR

Total DNA (genomic + mitochondrial) was extracted from heart tissue using a QIAamp DNA Mini Kit (Qiagen). The relative mtDNA copy number was measured by PCR and corrected by simultaneous measurement of the nuclear DNA. The forward and reverse primers for mtDNA, which are complementary to the sequence of the mouse mitochondrial cytochrome c oxidase subunit II gene, were 5′-AA-CCATAAGGGCACCATGATAC-3′ and 5′-GGATGGCATTGTTTAAATCC-3′. 5′-CGGCGACGAC-CATTGGAAC-3′ and 5′-GAATCGAACCCTGTAT-TCCCGTGC-3′, sequences complementary to the 18S gene, were the primers used for the detection of nuclear DNA. Quantitative PCR was performed on an Eppendorf Realplex S2 (Eppendorf) and Mesa Blue qPCR Master Mix Plus for SYBR assay (Euorgenetic). Primers for mouse Ppargc1a [PGC-1α (peroxisome-proliferator-activated receptor γ co-activator-1α)], Mfn2 (mitofusin 2), Opa1 (optic atrophy 1 homologue), Drp1 (dynamin 1-like) and Fis1 (fission 1 homologue) (Table 1) were from Invitrogen. Actb (β-actin) was used as an internal control. Realplex Software was used to quantify differences in gene expression. Results are expressed as $2^{-\Delta \Delta Ct}$.

Statistics

Results were analysed with the SPSS for Windows software, version 11.0.1. Results represent means ± S.E.M. and were analysed by ANOVA procedures. When a significant difference was found, we identified specific differences between groups with a sequentially rejective Bonferroni procedure. After the application of the Bonferroni correction, $P < 0.05$ was taken as a level of statistical significance. Survival was evaluated with a Fisher exact test.

RESULTS

Ciclosporin treatment improved survival in doxorubicin-treated mice

Doxorubicin toxicity was evaluated in vivo using two different protocols of drug administration, i.e. protocols A and B (see the Materials and Methods section for details). Mice mortality was observed only in mice that received doxorubicin treatment. Doxorubicin-treated mice began to die between 4 and 5 days of follow-up in protocol A, and between 6 and 8 weeks in protocol B. The mortality rate was 55% at day 8 in protocol A and then remained stable till day 10. In protocol B, a maximal mortality rate of 60% was reached in the doxorubicin-treated group after 13 weeks. In both protocols A and B, CsA (1 mg·kg$^{-1}$ of body weight·48 h$^{-1}$) significantly improved survival rate after administration of doxorubicin (25 and 40% mortality rate in protocols A and B respectively; $P < 0.05$ compared with controls), whereas tacrolimus exerted deleterious effects on mortality rate (results not shown) in doxorubicin-treated mice.

Ciclosporin prevented doxorubicin-induced myocardial contractile dysfunction

At baseline, heart rate and LVFS were similar among the six groups of mice. At day 10 of protocol A, doxorubicin induced a significant left ventricular systolic dysfunction in mice compared with the control group ($P < 0.05$;
Table 1  Gene accession numbers and primer sequences

<table>
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<tr>
<th>GenBank® accession number</th>
<th>Gene name</th>
<th>Primer sequence (5′ → 3′)</th>
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<td>NM_008904</td>
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<td></td>
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<td>TGAGGACCCGCTACAGGATTTG</td>
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<td>NM_133752</td>
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<td>CTCGGGCTAACAGTACACCC</td>
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<tr>
<td>NM_152816</td>
<td>Drp1</td>
<td></td>
<td>GCGTTCCTAACTTCAGAAGC</td>
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<td>NM_025562</td>
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<td>NM_007393</td>
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<td>AGCTGCCTGACGGCCAGTGC</td>
<td>GCTCAGGAGGAGCGATAGC</td>
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</table>

Table 2  LVFS assessed by echocardiography in mice

Echocardiographic measurements were performed in triplicate for each mouse. LVFS was calculated in accordance with the American Society of Echocardiography guidelines. See the Materials and methods section for details of the doxorubicin (Doxo), CsA and tacrolimus (FK506) treatment groups. Results are means ± S.E.M. Results were analysed with one-way ANOVA and Bonferroni’s multiple comparison post-hoc adjustment (n = 12 in each group). *P < 0.05 compared with control.

<table>
<thead>
<tr>
<th>Group</th>
<th>At 1.5 weeks of doxorubicin</th>
<th>At 16 weeks of doxorubicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.40 ± 0.02</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>CsA</td>
<td>0.40 ± 0.03</td>
<td>0.38 ± 0.03</td>
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<tr>
<td>FK506</td>
<td>0.43 ± 0.05</td>
<td>0.40 ± 0.02</td>
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<tr>
<td>Doxo</td>
<td>0.31 ± 0.03*</td>
<td>0.28 ± 0.04*</td>
</tr>
<tr>
<td>Doxo + CsA</td>
<td>0.39 ± 0.04</td>
<td>0.35 ± 0.03</td>
</tr>
<tr>
<td>Doxo + FK506</td>
<td>0.32 ± 0.05*</td>
<td>0.29 ± 0.05*</td>
</tr>
</tbody>
</table>

Table 2). Treatment with CsA reduced doxorubicin-mediated myocardial dysfunction. Tacrolimus exerted no effect on left ventricular function of doxorubicin-exposed mice (Table 2). In protocol B, similar results were observed in the echocardiographic study at week 16, with the protective action of CsA against doxorubicin-related contractile dysfunction (Table 2).

Ciclosporin prevented doxorubicin-induced cardiac mitochondrial dysfunction

Respiration measurements were started immediately after the preparation of permeabilized cardiac fibres. The addition of cytochrome c (10 μM) had no significant effects on state 3 respiration (results not shown), suggesting that procedures used to prepare cardiac fibres did not damage the mitochondrial outer membrane. In this setting, doxorubicin altered oxidative phosphorylation respiration in a mouse model of cardiotoxicity, as reflected by reduced RCR (Table 3). Reduction of RCR induced by doxorubicin was the result of both reduced ADP-stimulated respiration and increased state 4 respiration (without ADP) with glutamate and malate as substrates in the mouse acute model. In this context, administration of CsA largely prevented doxorubicin-induced deterioration of mitochondrial respiration parameters at 10 days after doxorubicin administration in protocol A (Table 3). Doxorubicin cardiotoxicity was accompanied by mitochondrial membrane potential dissipation (Table 3), which was prevented by ciclosporin. The amount of calcium required to open the transition pore averaged 220 ± 18 μmol/l in isolated mitochondria from control mice, which corresponded to 220 nmol/mg of mitochondrial protein. A similar amount of calcium was required to open the pore in CsA-treated control groups. The calcium overload required to induce mitochondrial permeability transition was significantly reduced in the doxorubicin group, averaging 93 ± 25 μmol/l in mice mitochondria. Whereas tacrolimus had no effects, CsA in vivo treatment significantly increased the calcium amount required for pore opening after doxorubicin exposure (Table 3). In select experimental runs, in vitro pre-incubation with CsA, a known inhibitor of the MPTP, delayed calcium-induced calcium release in both control and doxorubicin mitochondria (results not shown). The findings that in vitro CsA increased calcium uptake capacity suggested that the loss of ability to take up further calcium correlated with the MPTP-dependent process.

Effects of ciclosporin on cardiac mitochondrial biogenesis and network dynamics in doxorubicin-treated mice

At day 10 post-doxorubicin treatment, mitochondrial ultrastructure analyses indicated increased mitochondrial density and decreased areas of individual mitochondria (Figure 1), suggesting an increased population of mitochondria and reduced mitochondrial size (Table 4). Compared with the controls, the percentage of...
Table 3  Mitochondrial functional studies in cardiac fibres and isolated mitochondria
See the Materials and methods section for details of the doxorubicin (Doxo), CsA and tacrolimus (FK506) treatment groups. State 4 respiration rate is determined with glutamate (5 mM) + malate (2 mM) without ADP. State 3 respiration rate is determined in the presence of ADP (2.5 mM) with mitochondrial substrates added to the preparation. RCR is the ratio between state 3 respiration rate over state 4. Assessment of mitochondrial membrane potential (ΔΨm, mV) and calcium pulse studies are performed in isolated mitochondria. External added calcium (nmol/mg of protein) represents the amount of externally added calcium necessary to trigger the massive calcium release, an indicator of mitochondrial permeability transition. Results are means ± S.E.M. Results were analysed with one-way ANOVA and Bonferroni’s multiple comparison post-hoc adjustment (n = 12 in each group). *P < 0.05 compared with control; †P < 0.05 compared with Doxo + CsA.

<table>
<thead>
<tr>
<th>Group</th>
<th>Rate of respiration (pmol O2·s⁻¹·mg⁻¹ of wet weight)</th>
<th>RCR</th>
<th>ΔΨm</th>
<th>External added calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>53 ± 2</td>
<td>213 ± 9</td>
<td>3.8 ± 0.5</td>
<td>205 ± 5</td>
</tr>
<tr>
<td>CsA</td>
<td>54 ± 4</td>
<td>231 ± 5</td>
<td>4.0 ± 0.2</td>
<td>210 ± 3</td>
</tr>
<tr>
<td>FK506</td>
<td>50 ± 4</td>
<td>205 ± 6</td>
<td>4.0 ± 0.6</td>
<td>200 ± 4</td>
</tr>
<tr>
<td>Doxo</td>
<td>65 ± 5*</td>
<td>138 ± 8*</td>
<td>2.3 ± 0.5*</td>
<td>183 ± 7*</td>
</tr>
<tr>
<td>Doxo + CsA</td>
<td>52 ± 3</td>
<td>195 ± 8*′</td>
<td>3.4 ± 0.6′†</td>
<td>206 ± 6</td>
</tr>
<tr>
<td>Doxo + FK506</td>
<td>70 ± 5*</td>
<td>120 ± 8*</td>
<td>2.31 ± 0.7*</td>
<td>173 ± 5*</td>
</tr>
</tbody>
</table>

Figure 1  Heart ultrastructure
Electron micrographs of the myocardium (same magnification; scale bar, 1 μm) in control mice (left-hand panel), showing normal myofibrillary and mitochondria structures, and doxorubicin-treated mice (middle panel). The mitochondria in the cardiac ventricles of doxorubicin-treated mice were more numerous and often significantly larger than control mouse hearts. In some myocytes, the marked mitochondrial proliferation appeared to have replaced the sarcomeric assembly. The right-hand panel represents the electron micrograph of a CsA- and doxorubicin-treated heart.

mitochondria with a size less than 1.5 μm was increased, whereas the percentage of enlarged mitochondria (mitochondria >1.5 μm) was reduced in doxorubicin-treated mice, suggesting increased mitochondrial fragmentation (Table 4). The mitochondrial biogenesis profile investigated by real-time quantitative PCR indicated a 2-fold increase in mtDNA content (Figure 2) and a 3-fold increase in Ppargc1α in doxorubicin-treated mice (Figure 2). The mitochondrial fusion and fission profile investigated by real-time quantitative PCR included a 4-fold increase in Mfn2, a 2-fold increase in Opa1 and a 2-fold increase in Fis1, whereas Drp1 was unchanged in doxorubicin-treated mice (Figure 3).

The increases in mtDNA content and mitochondrial biogenesis induced by doxorubicin were not prevented by CsA treatment (Figure 2). CsA largely prevented alterations in mitochondrial fusion/fission balance (Figure 3) and ultrastructural changes (Table 4) that were observed in doxorubicin-treated mice. Normalization of the mitochondrial fusion/fission balance together with the maintained mitochondrial biogenesis signal induced by CsA were associated with major improvements in respiratory capacity (Table 3). Tacrolimus, a calcineurin inhibitor that does not interact with mitochondrial targets, had no effects on mitochondrial respiration (Table 3), myocardial contractile performance and survival in doxorubicin-treated mice.

DISCUSSION
The results of our present study reveal the important findings that CsA, a potent inhibitor of mitochondrial transition pore, protected mice against acute and chronic doxorubicin cardiotoxicity. The ciclosporin derivative tacrolimus, which has no effects on mitochondria, exerted no such beneficial effects, suggesting that cardioprotection associated with CsA was related to its inhibitory property on the mitochondrial permeability transition. Treatment with CsA was accompanied by the preservation of cardiac bioenergetics and mitochondrial network organization, which could be involved in the observed cardioprotection against doxorubicin.

Doxorubicin increases the susceptibility of mitochondria to oxidative stress and calcium-induced mitochondrial permeability transition, which contributes...
Table 4: Mitochondrial ultrastructure analyses in mice heart
See the Materials and methods for details of the doxorubicin (Doxo) and CsA treatment groups. Mitochondrial size is the mean of 1000 individual mitochondrial cross-sectional diameter. Elongated mitochondria are mitochondria of more than 1.5 μm. The percentage of mitochondria of a size less than 1.5 μm was used as an index of mitochondrial fragmentation. Results are means ± S.E.M. (n = 8 hearts in each group). Results were analysed with one-way ANOVA and Bonferroni’s multiple comparison post-hoc adjustment. * P < 0.05 compared with control; † P < 0.05 compared with Doxo + CsA.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mitochondria density (μm² of mitochondrial/μm² of total tissue volume)</th>
<th>Mitochondrial size (μm)</th>
<th>Elongated mitochondria (% mitochondria number per area)</th>
<th>Fragmented mitochondria (% mitochondria number per area)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.38 ± 0.01</td>
<td>1.12 ± 0.04</td>
<td>20.2 ± 2.4</td>
<td>79.8 ± 2.4</td>
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<tr>
<td>CsA</td>
<td>0.39 ± 0.01</td>
<td>1.09 ± 0.02</td>
<td>20.3 ± 0.6</td>
<td>79.4 ± 0.6</td>
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<td>Doxo</td>
<td>0.47 ± 0.01*</td>
<td>0.94 ± 0.06*</td>
<td>7.7 ± 1.6*</td>
<td>92.3 ± 1.7*</td>
</tr>
<tr>
<td>Doxo + CsA</td>
<td>0.40 ± 0.01</td>
<td>1.10 ± 0.02</td>
<td>16.8 ± 1.4†</td>
<td>83.2 ± 1.4†</td>
</tr>
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</table>

Figure 2: Mitochondrial biogenesis assessment
Effects of doxorubicin (doxo) on mtDNA copy number (A) and Ppargc1a (PGC-1α) RNA expression (B). Results are expressed as means ± S.E.M. (n = 8 mice in each group). * P < 0.05 compared with matched control. See the Materials and methods section for details of the doxorubicin (doxo) and CsA treatment groups.

Figure 3: Mitochondrial dynamics profile
Effects of doxorubicin (doxo) on relative RNA levels of Mfn2 (A), Opa1 (B), Drp1 (C) and Fis1 (D). Results are expressed as means ± S.E.M. (n = 8 mice in each group). * P < 0.05 compared with matched control. See the Materials and methods section for details of the doxorubicin (doxo) and CsA treatment groups. CsA had no effects on control (results not shown).

The effects of doxorubicin administration in vivo on mitochondrial respiration can be restored by adding CsA in vitro, implicating mitochondrial permeability transition as a direct causative factor of doxorubicin on mitochondrial bioenergetics [7]. Similarly, the findings of the present study suggest that in vivo treatment with low doses of CsA may limit MPTP opening, mitochondrial potential loss and contractile depression in acute and chronic models of cardiac toxicity induced by doxorubicin [13,14]. Previous studies on the effects of CsA on cardiotoxicity have yielded conflicting results. Mice pretreated with high concentrations of ciclosporin (12.5 or 25 mg/kg of body weight) are more vulnerable to doxorubicin toxicity [16,17]. Deleterious effects of high doses of ciclosporin have been related to inhibition of the membrane transport protein P-glycoprotein, which acts as an energy-dependent pump able to increase doxorubicin cellular efflux and reduce its intracellular retention [16,17]. In contrast, low doses of ciclosporin (0.5–2 mg/kg of body weight), which have no inhibitory effects on P-glycoprotein, may decrease doxorubicin-induced myocardial damage, i.e. elevation of serum LDH and CPK-MB enzymes [15]. Our results are consistent with studies showing that low-dose ciclosporin can protect the myocardium in the context of cardiac injury induced by doxorubicin. Interestingly, beneficial effects of the MPTP inhibitor CsA have been shown to generate a similar protection in acute myocardial infarction patients [18]. Mechanisms by which CsA can prevent doxorubicin-induced cardiotoxicity involve alteration of mitochondrial transmembrane potential through its inhibitory property on mitochondrial permeability transition [3,7,13,14]. Although significant and consistent with our previous studies [13,14], the decrease in membrane potential observed in mitochondria prepared...
from doxorubicin-treated mice could not be sufficient to directly compromise ATP synthesis. For example, it has been proposed that the ATP synthesis kinetics is a sigmoid function of membrane potential that reaches saturation at approx. \((-100\) mV [19].

One possible explanation for the protective effects of CsA may be associated with the preservation of a healthy mitochondrial network, due to mitochondrial dynamics. Mitochondrial dynamics refers to repetitive cycles of fusion and fission between mitochondria that are controlled by GTPases involved in fusion, Opal and Mfn2, and the fission protein Fis1 [20,21]. These opposing processes determine the architecture of the entire mitochondrial population and influence mitochondrial functions, including respiration and calcium buffering [22–24]. Mitochondrial transmembrane potential has been critically implicated in the regulation of mitochondrial dynamics, with depolarized mitochondria being the substrate for mitochondrial autophagy [22,23]. In our present study, mitochondrial dynamics were altered, as doxorubicin increased mRNA expression of GTPases involved in mitochondrial fusion, Opal and Mfn2, and proteins involved in mitochondrial fission, such as Fis1. This new information is important as derangements of mitochondrial dynamics play a critical role in the failing heart [25]. The findings that CsA normalized RNA expression of GTPases involved in fusion, Opal and Mfn2, and the fission protein Fis1, suggest that changes in mitochondrial membrane potential can modulate processes that govern mitochondrial dynamics. In agreement, recent findings in an embryonic cardiac cell line have shown that CsA blocked the dissipation of membrane potential and changes in Opal expression that occurred during simulated ischaemia in H9c2 cell lines [25]. Overall, it may be proposed that dissipation of the mitochondrial membrane potential associated with doxorubicin cardiotoxicity may induce mitochondrial network fragmentation and subsequent mitochondrial dysfunction. In this context, stabilization of membrane potential by CsA would result in mitochondrial bioenergetics preservation and improved cardiac function.

An alternative explanation for the beneficial effects of CsA on doxorubicin-induced mitochondrial dysfunction may be changes in heart mitochondrial biogenesis. Previous studies reported that doxorubicin may elicit mtDNA polymerase activation, DNA repair and replication, and mitochondrial proliferation in response to heart oxidative mitochondrial damage [26,27]. In sharp contrast, mitochondrial biogenesis inhibition has also been found in acute models of doxorubicin cardiotoxicity [9,28]. In our model, we observed that doxorubicin administration was accompanied with heart mitochondrial proliferation and increased DNA content, suggesting mitochondrial biogenesis stimulation. In the hearts of doxorubicin-treated mice, CsA did not alter mitochondrial biogenesis, DNA content and PGC-1α protein expression, even though the mitochondrial content, i.e. density and size, was normalized. Interpretation of these conflicting results is not readily evident as the effects of CsA on mitochondrial biogenesis are largely unknown. Calcineurin has been shown to increase Ppargc1a gene transcription, and overexpression of constitutively active calcineurin in skeletal muscle of transgenic mice results in increased expression of PGC-1α [29,30]. Furthermore, expression of constitutively active calcineurin in cardiac myocytes has been shown to result in increased expression of PGC-1α and the induction of a wide range of genes involved in mitochondrial energy metabolism [30]. In this context, inhibition of calcineurin with CsA did not prevent increases in PGC-1α and mitochondrial proteins [31]. These results are consistent with the absence of effects of CsA on increased DNA content and PGC-1α protein expression in our model.

Overall, doxorubicin is an anthracycline antibiotic that is widely used in the clinical setting as a major chemotherapeutic agent, yet its usefulness is limited by cardiac toxicity. Unfortunately, several strategies aimed at ameliorating this cardiotoxicity (i.e. limitation of the cumulative dose of anthracyclines, different anthracycline dosage schedules and use of different antioxidant agents) have provided only modest efficacy. As a different hypothesis, our studies suggest that, in animal and human myocardium, low doses of CsA can prevent doxorubicin-induced mitochondrial and myocardial contractile dysfunction through its inhibitory effects on mitochondrial permeability transition [13,14]. Such beneficial effects of CsA have been demonstrated in patients suffering from acute myocardial infarction with promising results on infarct size [18].

In conclusion, our study suggests that mitochondrial membrane potential stabilization with CsA prevents doxorubicin-induced heart contractile dysfunction through, at least in part, modulation of mitochondrial bioenergetics.

**AUTHOR CONTRIBUTION**

Xavier Marechal, David Montaigne and Camille Marciniak were responsible for data collection and analysed the data. All authors revised the final paper. Philippe Marchetti was responsible for the mitochondrial membrane potential data collection and analyses, and reviewing the final version of the paper. Jean Claude Beauvillain was responsible for transmission electronic microscopy and reviewing the paper. Steve Lancel was responsible for statistical analysis and revising the paper. Sidi Mohamed Hassoun was responsible for revising the paper. Remi Nievrie was responsible for data collection, analysing the data and writing the paper.
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