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**Cardioprotection by the TSPO ligand 4'-chlorodiazepam is associated with inhibition of mitochondrial accumulation of cholesterol at reperfusion.**

Stéphanie PARADIS<sup>1,2</sup>, Valerio LEONI<sup>3</sup>, Claudio CACCIA<sup>3</sup>,

Alain BERDEAUX<sup>1,2</sup>, Didier MORIN<sup>1,2</sup>

<sup>1</sup> Inserm, U955, Equipe 3, Créteil, 94000, France.

<sup>2</sup> Université Paris Est, Faculté de Médecine, Créteil, 94000, France.

<sup>3</sup> Laboratory of Clinical Pathology and Medical Genetics, Foundation IRCCS Neurology Institute Carlo Besta, Milano, Italy.

Corresponding author:

Didier Morin, Faculté de Médecine de Créteil, INSERM U955-équipe 03, 8 rue du Général Sarrail, 94010 Créteil Cedex, France.

Tel : +33 (0) 1 49813661. Fax: +33 (0) 1 49813661. Email: didier.morin@inserm.fr

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**Abstract**

*Aims:* The translocator protein (TSPO) is located on the outer mitochondrial membrane where it is responsible for the uptake of cholesterol into mitochondria of steroidogenic organs. TSPO is also present in the heart where its role remains uncertain. We recently showed that TSPO ligands reduced infarct size and improved mitochondrial functions after ischemia-reperfusion. This study thus sought to determine whether cholesterol could play a role in the cardioprotective effect of TSPO ligands.

*Methods and Results:* In a model of 30 min coronary occlusion/15 min reperfusion in Wistar rat, we showed that reperfusion induced lipid peroxidation as demonstrated by the increase in conjugated diene and thiobarbituric acid reactive substance formation and altered mitochondrial function (decrease in oxidative phosphorylation and increase in the sensitivity of mitochondrial permeability transition pore opening) in *ex-vivo* isolated mitochondria. This was associated with an increase in mitochondrial cholesterol uptake ( $89.5 \pm 12.2$  vs  $39.9 \pm 3.51$  nmoles/mg protein in controls,  $p < 0.01$ ) and a subsequent strong generation of auto-oxidized oxysterols, i.e.  $7\alpha$ - and  $7\beta$ -hydroxycholesterol, 7-ketocholesterol, cholesterol- $5\alpha,6\alpha$ -epoxide and  $5\beta,6\beta$ -epoxide (+173%, +149%, +165%, +165% and +193% vs controls, respectively;  $p < 0.01$ ). Administration of the selective TSPO ligand 4'-chlorodiazepam inhibited oxidative stress, improved mitochondrial function, and abolished both mitochondrial cholesterol accumulation and oxysterol production. This was also observed with the new TSPO ligand TRO40303.

*Conclusions:* These data suggest that 4'-chlorodiazepam inhibits oxidative stress and oxysterol formation by reducing the accumulation of cholesterol in the mitochondrial matrix at reperfusion and prevents mitochondrial injury. This new and original mechanism may contribute to the cardioprotective properties of TSPO ligands.

## 1. Introduction

It is well established that one of the major causes of cell death during myocardial ischemia-reperfusion is mitochondrial dysfunction resulting from an increase in the permeability of mitochondrial membranes<sup>1</sup> at reperfusion. Several mechanisms, which are not mutually exclusive, have been suggested to be involved in this process<sup>2</sup> but formation of a multiprotein complex involving both the inner and the outer membranes, named the “mitochondrial permeability transition pore” (mPTP) is now considered as a major event in the induction of myocardial cell death. Thus the limitation of mPTP opening following ischemia-reperfusion is a major target for cardioprotection.<sup>3</sup>

The translocator protein (18kDa) or TSPO is located on the outer mitochondrial membrane where it has been shown to interact with proteins implicated in mPTP formation and to potentially regulate the pore.<sup>4,5</sup> A broad spectrum of putative functions has been proposed for TSPO but the best characterized is the uptake of cholesterol into mitochondria of steroidogenic cells. Accordingly, TSPO is widely expressed in steroidogenic tissues where its activity is a limiting step for steroid hormone synthesis. TSPO has also been found in non-steroidogenic tissues where its role remains uncertain. In these tissues, TSPO has been implicated in numerous biological functions such as respiration, reactive oxygen species production, apoptosis or inhibition of cell proliferation<sup>5,6</sup> but TSPO ligands were also found to modify the intracellular distribution of cholesterol in non-steroidogenic cells.<sup>7</sup> TSPO is abundant in the heart and we and others have previously shown that the TSPO ligand 4'-chlorodiazepam reduced infarct size after ischemia-reperfusion.<sup>8-10</sup> Recently, we observed a similar cardioprotective effect with a new TSPO ligand TRO40303<sup>11</sup> which binds specifically to the cholesterol site of TSPO, confirming the interest of this pharmacological target. The cardioprotective effect of these drugs was associated with limitation of mitochondrial membrane permeability and stabilization of mitochondrial function. Alternatively, it has also

been suggested that mitochondrial cholesterol variations could alter mitochondrial function by uncoupling oxidative phosphorylation and decreasing ATP synthesis during ischemia-reperfusion.<sup>12</sup> It was also reported that induction of mPTP opening was accompanied by an increase in mitochondrial membrane fluidity due to conformational changes of mPTP forming proteins.<sup>13</sup> Given that cholesterol is a major factor determining the physical properties of membranes, we hypothesized that the cardioprotective effect of TSPO ligands could be associated with change in mitochondrial cholesterol concentrations. To investigate this possibility we evaluated the cholesterol variations in mitochondria in a rat model of myocardial ischemia-reperfusion in the absence or in the presence of the TSPO ligands 4'-chlorodiazepam and TRO40303.

## **2. Methods**

A detailed description of the methods can be found in the Supplementary material online.

Brief descriptions are as follows.

### **2.1. Animals**

All animal procedures used in this study were in strict accordance with the Directives of the European Parliament (2010/63/EU-848 EEC) and recommendations of the French Ministère de l'Agriculture. Animal experiments were approved by the Institutional Animal Care and Use Committee of Paris-Est University (approval number: 08/03/11-05). Male Wistar rats, weighting 250 to 300 g (7-8 weeks), were purchased from Janvier (Le Genest-St-Isle, France). They were housed in a room maintained under constant environmental conditions (temperature 22-25°C and a constant cycle of 12-h light/dark). They were acclimatized to the animal room before being used and received standard rat diet and water ad libitum.

## 2.2. In vivo coronary artery occlusion-reperfusion

Rats were anesthetized with one administration of sodium pentobarbital (60 mg/kg i.p.) and then intubated and ventilated with room air using a respirator (model 683; Harvard Apparatus Inc.). The depth of anesthesia was monitored using the tail pinching response and the pedal reflex. A left thoracotomy was performed at the fourth intercostal space. A surgical needle was passed under the left main coronary artery and the ends of the suture were passed through a polypropylene tube to form a snare. Tightening the snare induced coronary artery occlusion, and releasing the ends of the suture initiated reperfusion. In a first group of rats, the coronary artery was occluded for 30 min. In the other groups, the coronary artery was occluded for 30 min and released for 15 min or 2 h of reperfusion, except in the sham group in which the surgical procedure was identical to others but the coronary artery was not occluded. The area at risk was conservatively estimated by the position of the suture. A 15 min reperfusion was used for extracting myocardial mitochondria and determining oxidative phosphorylation, mPTP opening, mitochondrial membrane fluidity, conjugated diene generation and mitochondrial cholesterol concentrations. This reperfusion time was chosen because mPTP opening was shown to occur within the first minutes of reperfusion.<sup>14</sup> A 2 h reperfusion was used for extracting cardiac mitochondria and determining lipid peroxidation. 4'-Chlorodiazepam (10 mg/kg), TRO40303 (3 mg/kg) or their vehicle (N,N-dimethylformamid for 4'-chlorodiazepam and intralipid for TRO40303) were administered as a 5 min infusion through the jugular vein. In the ischemia-reperfused groups, 4'-chlorodiazepam and TRO40303 or their vehicle were administered 10 min before coronary artery occlusion and 10 min before reperfusion, respectively. When the drugs were administered to the sham groups, they were infused 25 min before heart excision.

At the end of the reperfusion, the left ventricle from sham rats (noninfarcted) or from the area at risk of rats that underwent ischemia-reperfusion was excised to prepare mitochondria.

### **2.3. Isolation of mitochondria and separation of mitochondrial membranes and matrix**

Mitochondria were isolated by differential centrifugation as described previously.<sup>15</sup> To separate the mitochondrial membranes (inner plus outer) and the matrix (intermembrane space and matrix), Triton X-100 (0.1%) was added to the mitochondrial suspensions which were subjected to three cycles of freezing/thawing (10 min at -80°C/ 5 min at 30°C). The mitochondria were then centrifuged at 100,000 g for 1 h at 4°C. The supernatants corresponded to the matrix. The pellets, corresponding to the membranes, were resuspended in the MSH buffer and centrifuged at 50,000 g for 10 min at 4°C. The protein concentrations were approximately 4-5 mg and 10-12 mg protein/ml for the matrix and the membranes, respectively, and were determined using the “Advanced protein assay reagent” (Sigma, catalogue number 57697).

### **2.4. Cholesterol loading in isolated mitochondria**

Isolated mitochondria (5 mg protein/ml) were incubated with a 1.5 µl cholesterol (10 g/l)/bovine serum albumin (300 g/l) mixture<sup>16</sup> for 30 min at 4°C under low agitation. The mitochondria were then washed three times in the MSH buffer and centrifuged at 15,000 g for 5 min.

### **2.5. Mitochondrial oxygen consumption and calcium-induced mPTP opening**

The consumption of oxygen of isolated mitochondria was measured as described previously.<sup>17,18</sup> mPTP opening was assessed by monitoring mitochondrial calcium retention capacity. Cardiac mitochondria (1 mg /ml) energized with 2.5 mM pyruvate/ 2.5 mM malate were incubated at 30°C in the respiration buffer, including 3 µM of the calcium green-5N fluorescent probe (Molecular Probes, Invitrogen). The reaction was started by addition of successive 10 µM calcium pulses. After each addition, a rapid uptake was observed followed

by a dynamic steady state corresponding to the equilibrium between the influx and the efflux of calcium. When the maximal calcium loading threshold was reached, this equilibrium was disrupted, and calcium was released. The concentration of calcium in the extra-mitochondrial medium was monitored by means of a Perkin Elmer LS 50B fluorescence spectrometer at excitation and emission wavelengths of 506 and 532 nm, respectively. The calcium signal was calibrated by addition of known calcium concentrations to the medium.

## **2.6. Fluorescence anisotropy**

Fluidity of mitochondrial membranes was evaluated by fluorescence anisotropy of mitochondria-bound dyes 1,6-diphenyl-1,3,5-hexatriene (DPH) and hematoporphyrin IX (HP). DPH and HP interact with hydrophobic lipid phases and protein sites of biological membranes, respectively.<sup>13</sup> The DPH or HP labeling was achieved by the addition of 1  $\mu$ M into stirred mitochondria suspension (0.2 mg/ml). The mixture was incubated for 10 min or 5 min before measuring anisotropy for DPH or HP, respectively. DPH was excited at 340 nm and its fluorescence was detected at 460 nm whereas HP was excited at 520 nm and its fluorescence was detected at 626 nm with a Perkin Elmer LS 50B fluorescence spectrometer. Steady-state fluorescence anisotropy values were obtained by measurements of the fluorescence intensity polarized parallel (IVV) and perpendicular (IVH) to the vertical plane of polarization of the excitation beam. The fluorescence anisotropy ( $r$ ) is defined by the equation  $r = (IVV - GIVH)/(IVV + 2GIVH)$ , where  $G$  equals  $IHV/IHH$  and is the correction factor for instrumental artefacts.<sup>19</sup>

## **2.7. Assay of lipid peroxidation and conjugated diene identification in mitochondrial membranes**

Conjugated dienes were determined as described by Recknagel and Glende<sup>20</sup> and lipid peroxidation was assessed as the generation of thiobarbituric acid reactive substances (TBARs).<sup>21</sup>

### **2.8. Lipid extraction, determination of mitochondrial cholesterol and oxysterol levels**

Whole mitochondria, membranes and matrix samples (5 mg protein/ml) were added to a chloroform-Triton X-100 1% mixture (v:v). After 1 min of strong agitation, the samples were centrifuged at 8,000 g for 5 min at room temperature. The upper phases were discarded and the lower phases containing cholesterol were evaporated for 3 h at 60°C. The total cholesterol of whole mitochondria, membrane and matrix samples was determined with a commercial kit (Amplex red cholesterol assay kit, Invitrogen). Oxysterols were assayed in mitochondria extracts by isotope dilution-mass spectrometry as described previously.<sup>22</sup>

### **2.9. Enzymatic assays**

All enzymatic activities were determined by spectrophotometric methods using a Jasco model V-530 spectrophotometer and were shown to be linear in the protein concentration range used. Citrate synthase activity was determined at 412 nm by measuring the initial rate of reaction of liberated coenzyme A-SH with 5, 5'-dithio-bis-2-nitrobenzoic acid. Mitochondria (2.5 µg/ml) were incubated in a buffer (KH<sub>2</sub>PO<sub>4</sub> 10 mM, MgCl<sub>2</sub> 2 mM, EDTA 1 mM, pH 7.4 at 37°C) supplemented with 0.2% Triton X-100 and containing 0.5 mM acetyl-CoA and 0.1 mM 5, 5'-dithio-bis-2-nitrobenzoic acid in a final volume of 1 ml. The reaction was carried out at 37°C and initiated by the addition of 0.5 mM oxaloacetate.

The determination of monoamine oxidase activity was performed according to the method of Bembek et al.<sup>23</sup> using kynuramine as a substrate and monitoring the formation of 4-hydroxyquinoline at 316 nm. Cytochrome C oxidase activity was assayed at 37°C according to

the method of Rustin et al.<sup>24</sup> by monitoring the oxidation of ferrocytochrome C (prepared from type III horse heart cytochrome C (Sigma)) at 550 nm.

## **2.10. Data analysis**

The data are reported as mean  $\pm$  S.E.M. Statistical significance was determined using either Student's two-tailed unpaired *t*-test or one-way analysis of variance (ANOVA) followed by Scheffe's post test. Significance was accepted when  $p < 0.05$ .

## **3. Results**

### **3.1. Myocardial ischemia-reperfusion affects mitochondrial function and mitochondrial cholesterol concentrations**

We first investigated the effect of ischemia-reperfusion on mitochondrial function. Our data confirmed that ischemia-reperfusion inhibited oxidative phosphorylation and sensitized mitochondria to mPTP opening. Indeed, 30 min ischemia followed by 15 min reperfusion decreased both ADP-stimulated respiration (state 3) and the respiratory control ratio (-33.7% versus sham) and significantly reduced the capacity of mitochondria to retain calcium before mPTP opening (-50.1% versus sham) (*Table 1*).

Using the same protocol we examined then the effect of ischemia-reperfusion on the mitochondrial concentration of cholesterol. In mitochondria isolated from control hearts the concentration of cholesterol was  $39.9 \pm 3.51$  nmoles/mg protein (*Figure 1*), a similar level to that previously obtained in rat and pig heart mitochondria.<sup>25,26</sup> There was no difference between free and total cholesterol showing the absence of esterified cholesterol and that cholesterol was essentially in the free form confirming previous observations by other investigators.<sup>25,26</sup> We also evaluated the concentration of cholesterol in isolated mitochondria subfractionated into membranes (inner plus outer) and matrix plus intermembrane space

(called matrix). *Figure 2* shows that the subfractionation method allowed us to obtain enriched fractions in mitochondrial membranes and matrix as demonstrated by the measure of the activity of specific enzymatic markers and *figure 1* also shows that cholesterol was present in both mitochondrial fractions.

Ischemia induced a decrease in mitochondrial cholesterol which is observed in both the membrane and the matrix fractions (*Figure 1*). Conversely, 15 min reperfusion produced a large increase in mitochondrial cholesterol concentration (+124% and +228% compared to sham and ischemic values, respectively). This increase mainly took place in the matrix fraction (*Figure 1*).

These changes in cholesterol concentrations were not due to variations of mitochondrial protein concentrations as citrate synthase activity was unchanged after ischemia and/or reperfusion (*Figure 1*). This confirms that the same quantity of mitochondria with intact membranes were included in the assays.

### **3.2. Myocardial ischemia-reperfusion alters mitochondrial membrane fluidity**

Because cholesterol enrichment can modify membrane fluidity, we examined the effect of ischemia-reperfusion on mitochondrial membrane fluidity. To this end, mitochondria were isolated from rat hearts subjected to 30 min ischemia followed by 15 min reperfusion and membrane fluidity was determined by measuring the change in the steady-state fluorescence anisotropy of two fluorescent probes bound to mitochondria, DPH and HP.

Ischemia-reperfusion caused an increase in the fluorescence anisotropy of DPH indicating a decrease in the membrane fluidity of lipid regions (*Table 2*). By contrast, a decrease in anisotropy was observed for HP-labelled mitochondria following ischemia-reperfusion.

In order to determine whether this change in membrane fluidity could be due to a direct effect of cholesterol on mitochondrial membranes, mitochondria isolated from control hearts were

loaded with cholesterol (see paragraph 2.4) which allowed to obtain a three-fold increase in cholesterol concentration in the mitochondrial membranes and not in the matrix according to Colell et al.<sup>27</sup>. Mitochondria loaded with cholesterol showed a higher fluorescence anisotropy of DPH than control mitochondria (*Table 2*). This confirms that a cholesterol membrane overload induces a decrease in membrane fluidity in accordance with previous reports.<sup>27,28</sup> In contrast to the results obtained with DPH, no change in anisotropy was observed when cholesterol loaded mitochondria were labelled with HP. This indicates that a high cholesterol level does not modify the arrangement of mitochondrial membrane proteins in isolated mitochondria and thus is not responsible for the decrease in anisotropy observed for HP-labelled mitochondria following ischemia-reperfusion.

### **3.3. The decrease in mitochondrial membrane fluidity may be related to lipid peroxidation**

Although membrane fluidity seems to be related to the variations of cholesterol in *in vitro* overloaded mitochondria, our findings demonstrate that no relation exists between the variations of mitochondrial cholesterol concentrations and membrane fluidity during ischemia-reperfusion. Indeed, the increase in mitochondrial cholesterol concentrations was observed in the matrix and not in the membranes. It is well established, however, that ischemia-reperfusion gives rise to an important reactive oxygen species production and thus to lipid peroxidation which has been shown to modify membrane fluidity.

We observed the formation of conjugated dienes in mitochondrial membranes after 15 min reperfusion and the release of TBARs after 2 h reperfusion (*Table 2*). This might explain the modifications of membrane fluidity.

### **3.4. 4'-chlorodiazepam inhibits lipid peroxidation, decreases mitochondrial cholesterol concentrations and improves mitochondrial function at reperfusion**

To determine whether TSPO might be involved in the accumulation of cholesterol in mitochondria at reperfusion, we examined the effect of treatment with the TSPO ligand 4'-chlorodiazepam in rats subjected to 30 min ischemia followed by 15 min reperfusion. 4'-chlorodiazepam was administered 10 min before ischemia at a dose (10 mg/kg) shown to be cardioprotective in a previous study.<sup>8</sup> *Figure 3* shows that 4'-chlorodiazepam strongly inhibited ischemia-reperfusion-induced mitochondrial cholesterol accumulation whereas it had virtually no effect on mitochondrial cholesterol concentration in sham operated rats when cholesterol was measured in whole mitochondria or in the matrix. We only found a decrease in cholesterol in the membrane fractions which was not observed in whole mitochondria probably because membrane cholesterol fractions only represent a small part of total mitochondrial cholesterol. It should be noted that this decrease was also observed after ischemia-reperfusion. A similar inhibiting effect on cholesterol accumulation at reperfusion was obtained following administration of the new TSPO ligand TRO40303 (*Figure 3*) that was also characterized as a cardioprotective agent.<sup>11</sup>

Interestingly, after ischemia-reperfusion, 4'-chlorodiazepam concomitantly inhibited lipid peroxidation, as demonstrated by the decrease in the conjugated diene and TBARs formation (*Table 2*), improved respiration parameters and decreased the sensitivity of mPTP opening (*Table 1*). This was associated with the restoration of membrane fluidity of DPH-loaded mitochondria, i.e. of membrane lipid regions. By contrast, no variation of membrane fluidity was observed with 4'-chlorodiazepam when mitochondria were loaded with HP although the sensitivity of mPTP opening decreased.

These findings suggest that 4'-chlorodiazepam inhibits matrix accumulation of cholesterol at reperfusion and improves mitochondrial function. It is also likely that TSPO is involved in

cholesterol transport into mitochondrial matrix in the heart and that cholesterol might contribute to the deleterious effects of ischemia-reperfusion. This hypothesis seems to be supported by the fact that TRO40303 also inhibits cholesterol accumulation induced by ischemia-reperfusion while neither drug changed cholesterol levels in mitochondria from sham-operated hearts.

### **3.5. 4'-chlorodiazepam prevents the formation of mitochondrial oxysterols**

Oxysterols (oxidized derivatives of cholesterol) are formed by cholesterol autoxidation in presence of oxidative stress and lipid peroxidation. Oxysterols have been suggested to induce apoptosis in different cellular models.<sup>29</sup> We hypothesized that they could provide a link between the impairment of mitochondrial function, the accumulation of cholesterol and the protective effect of TSPO ligands at reperfusion. Therefore, we examined the formation of the most commonly detected oxysterols resulting from the auto-oxidation of cholesterol, namely 7 $\alpha$ - and 7 $\beta$ -hydroxycholesterol, 7-ketocholesterol, cholesterol-5 $\alpha$ ,6 $\alpha$ -epoxide and 5 $\beta$ ,6 $\beta$ -epoxide. Oxysterol levels were quantified in sham, ischemia-reperfused and ischemia-reperfused 4'-chlorodiazepam-treated rats. As shown in *figure 4*, oxysterols were detected in cardiac mitochondria from sham animals, the highest concentration values being measured for 7 $\alpha$ - and 7 $\beta$ -hydroxycholesterol. Reperfusion of the ischemic myocardium increased the concentrations of oxysterols in comparison to control hearts. The administration of 4'-chlorodiazepam before ischemia-reperfusion totally abolished this effect. A similar effect was observed after TRO40303 administration.

## **4. Discussion**

TSPO has been implicated in numerous biological functions,<sup>6</sup> especially in the regulation of mitochondrial transport of cholesterol in steroid-producing tissues.<sup>30</sup> In other tissues, such as

the heart, its role remains uncertain. Several studies however have shown that TSPO ligands have cardioprotective properties in different models of myocardial infarction. Leducq et al.<sup>31</sup> reported that SSR180575 reduced the infarct size in rabbit isolated hearts and in anesthetized rats. More recently, it was shown that 4'-chlorodiazepam reduced cardiac injury and improved cardiac functional recovery during ischemia-reperfusion in rats.<sup>8,10</sup> We found similar results with a new TSPO ligand TRO40303<sup>11</sup> which is presently in a phase II clinical study in patients undergoing angioplasty to treat an acute myocardial infarction.<sup>32</sup> All these studies concluded that TSPO ligands exert their protective effect through preservation of mitochondrial function and prevention of apoptosis. In particular, TSPO ligands inhibited both oxidative stress and the increase in mitochondrial membrane permeability, specifically by limiting mPTP opening after ischemia-reperfusion. This is in line with several other studies suggesting an antiapoptotic function of TSPO ligands in different tissues. For example, SSR180575 decreased oxidative stress and apoptosis during cardiac ischemia-reperfusion<sup>31</sup> and protected polymorphonuclear leukocytes against TNF $\alpha$ -induced apoptosis in whole blood.<sup>33</sup> In the same way, 4'-chlorodiazepam protected lymphoblastoid and neuronal cells against TNF $\alpha$  and platelet-activating factor induced apoptosis, respectively.<sup>33,35</sup> 4'-Chlorodiazepam also prevented isoproterenol-induced cardiac hypertrophy and this was associated with an attenuation of isoproterenol-induced lipid peroxidation and changes in endogenous antioxidants.<sup>36</sup>

Despite these observations, the definite mechanism by which TSPO ligands could limit oxidative stress, improve mitochondrial function and reduce infarct size during ischemia-reperfusion remains unknown. It should be noted, however, that 4'-chlorodiazepam is able to inhibit the mitochondrial inner membrane anion channel, to decrease the incidence of arrhythmia and to have beneficial effects in reducing calcium overload which may contribute to its cardioprotective effect.<sup>9</sup>

We present here new findings suggesting that modulation of uncontrolled mitochondria cholesterol accumulation may be an additional mechanism explaining the cardioprotective effect of TSPO ligands. We found that reperfusion of an ischemic myocardium is associated with a rapid increase in mitochondrial cholesterol and oxysterol concentrations. This increment took place in the mitochondrial matrix and was not associated with an incorporation of cholesterol in membranes. Thus, the decrease in membrane fluidity observed in the lipid regions of mitochondrial membranes does not seem to be related to the change in cholesterol level but should be ascribed to oxidative stress.

Myocardial ischemia and reperfusion is accompanied by a burst of reactive oxygen species generation in mitochondria<sup>37</sup> and the influx of cholesterol into mitochondrial matrix offers a high quantity of cholesterol that is highly sensitive to reactive oxygen species attack for oxidation. When reactive oxygen species mediate free radical oxidation of cholesterol, a very complex mixture of oxysterols is formed in the cell, resulting from both the initial oxygenated species of cholesterol and their subsequent metabolites.<sup>38</sup> We observed the same behavior in cardiac mitochondria as a strong increase in concentration of auto-oxidized oxysterols, i.e. 7 $\alpha$ - and 7 $\beta$ -hydroxycholesterol, 7-ketocholesterol, and cholesterol-epoxides was obtained after ischemia-reperfusion. These metabolites are products of 7-hydroperoxycholesterol, which is formed from the oxidation of cholesterol through reactions initiated by free radical species, such as those arising from the superoxide/hydrogen peroxide/hydroxyl radical system.<sup>39</sup> 7 $\alpha$ -, 7 $\beta$ -Hydroxycholesterol and 7-ketocholesterol are products of radical reactions on cholesterol and products of lipid peroxidation. They have a toxic and metabolic effect<sup>37,40</sup> but they are not themselves propagators of lipid peroxidation. The presence of these oxysterols is a confirmation of oxidative stress and cholesterol auto-oxidation at reperfusion. 7-hydroperoxycholesterol, cholesterol-epoxides and the cholesterol radical from which they are derived, can propagate lipid peroxidation and exert their activity through a modification of the

biophysical organization of both lipids and proteins within the membranes.<sup>38</sup> To the best of our knowledge the possible role of oxysterols in the deleterious effects of myocardial ischemia-reperfusion has not been described up to now but accumulation of oxysterols has been shown to cause cell death in various cell lines by increasing oxidative stress.<sup>41</sup> In particular, oxysterols are involved in the development of atherosclerosis and in the genesis of neurodegenerative diseases<sup>29</sup> and it had been suggested that they may be biomarkers in these diseases.<sup>42</sup> 7-Ketocholesterol and 7 $\beta$ -hydroxycholesterol induce down-regulation of anti-oxidative defenses<sup>43</sup> and most of the toxic effects of oxysterols have been attributed to their ability to induce apoptosis through the mitochondrial pathway.<sup>29</sup>

In the present study we show that reperfusion induces lipid peroxidation of mitochondrial membranes as demonstrated by the increase in conjugated dienes and TBARs. This may be due to the oxidation of cardiolipin which is highly concentrated in mitochondrial membranes.<sup>44</sup> Indeed, cardiolipin is susceptible to peroxidation reactions due to its high content of unsaturated fatty acids and is emerging as an important factor in mitochondrial dysfunction as well as in the initial phase of the apoptotic process. Cardiolipin oxidation is thought to promote mPTP opening and cytochrome c release and prevention of cardiolipin peroxidation has been shown to protect hearts from reperfusion injury.<sup>45</sup>

In our study, 4'-chlorodiazepam inhibits lipid peroxidation, reduces the accumulation of cholesterol and abolishes the formation of oxysterols. This suggests that TSPO is responsible for the transport of cholesterol in cardiac mitochondria and highlights a possible relationship between the accumulation of cholesterol, its transformation to oxysterols and the oxidation of mitochondrial membranes during reperfusion of an ischemic myocardium.

On the basis of the results presented here, we suggest that the increased amount of cholesterol and oxysterols in mitochondrial matrix could contribute to mPTP opening and to an

inefficient oxidative phosphorylation offering reactive oxygen species to self sustaining lipid peroxidation.

As summarized in *figure 5*, by inhibiting cholesterol uptake into mitochondria at reperfusion, 4'-chlorodiazepam might prevent the accumulation of oxysterols favoring the improvement of oxidative phosphorylation and the inhibition of mPTP. This might be one mechanism by which TSPO ligands exert an indirect antioxidant effect and induce cardioprotection.

It should be noted that it has also been reported that elevated levels of cholesterol in mitochondria are associated with cellular resistance to apoptotic death.<sup>29</sup> This effect, however, was observed in conditions where cholesterol is accumulated in mitochondrial membranes, such as in cancer cells or in cells enriched in cholesterol, which differs from the acute conditions encountered during the reperfusion of the heart where cholesterol does not accumulate in membranes but rather in the matrix and is subjected to an oxidative stress.

One can argue that the concentration of oxysterols is very low compared to that of cholesterol and perhaps too low to induce toxic effects. In fact, the amount of oxysterols free in the mitochondrial matrix might be locally quite high compared to free cholesterol and this could explain a possible toxic effect. In addition several studies have revealed that 7 $\beta$ -hydroxycholesterol, 7-ketcholesterol and cholesterol-epoxides are cytotoxic at very low (nM) concentrations.<sup>46</sup>

Despite this study does not establish a cause-effect relationship between the accumulation of mitochondrial cholesterol/oxysterols and reperfusion damage, it clearly shows an association between both parameters. This opens to future investigations to further assess this relationship in details. However, in order to try to demonstrate the deleterious properties of oxysterols on mitochondria, we have performed experiments with isolated mitochondria loaded with commercially available oxysterols and found that up to 50  $\mu$ M oxysterols neither impaired oxidative phosphorylation nor the capacity of mitochondria to retain calcium. It has to be

noted that the *in vivo* situation could be different since oxysterols are likely to be produced by autoxidation of free cholesterol in the mitochondrial matrix whereas in our *in vitro* model they probably do not penetrate into the mitochondria. Therefore, no conclusion has been drawn from these experiments.

In conclusion, we found that myocardial ischemia-reperfusion induces an increase in mitochondrial cholesterol concentrations in the first minutes of reperfusion, leading to the production of oxysterols that may be involved in the deleterious mitochondrial effects of cardiac ischemia-reperfusion.

By inhibiting cholesterol accumulation in mitochondrial matrix, 4'-chlorodiazepam may inhibit oxysterol formation and prevent mitochondrial injury. This mechanism may contribute to the cardioprotection provided by TSPO ligands.

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**Conflict of interest:** none declared.

## **Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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## Figure legends

**Figure 1:** Effect of myocardial ischemia-reperfusion on mitochondrial cholesterol concentrations.

Rats were subjected to 30 min ischemia followed by 15 min reperfusion, then the hearts were excised and mitochondria were extracted for total cholesterol measurement. In parallel, citrate synthase activity was measured to determine the quantity of mitochondria in each group.

Each value represents the mean  $\pm$  S.E.M. of 6-8 independent experiments.

\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus corresponding sham value; ## $p < 0.01$ , ### $p < 0.001$  versus corresponding ischemia value.

**Figure 2:** Measurement of enzymatic activities.

For each marker, the activity was expressed as a percentage of the highest activity (100%) obtained. The maximal activities of the markers were  $15.1 \pm 0.95$ ,  $17.7 \pm 1.33$  and  $0.037 \pm 0.001$   $\Delta$ DO/min/mg protein for citrate synthase, cytochrome c oxidase and monoamine oxidase, respectively. Each value represents the mean  $\pm$  S.E.M. of 4-7 independent experiments.

\*\*\* $p < 0.001$  versus mitochondrial matrix.

**Figure 3:** 4'-Chlorodiazepam and TRO40303 inhibit the increase in mitochondrial cholesterol concentrations during myocardial ischemia-reperfusion.

Each value represents the mean  $\pm$  S.E.M. of 6-9 independent experiments.

\* $p < 0.05$ , \*\* $p < 0.01$  versus sham; # $p < 0.05$ , ### $p < 0.001$  versus ischemia-reperfusion (I/R).

**Figure 4:** 4'-Chlorodiazepam and TRO40303 prevent the formation of mitochondrial oxysterols during myocardial ischemia-reperfusion.

The basal sham values (i.e. 100%) for each oxysterol are as following (in pmoles/mg protein): 7 $\alpha$ -hydroxycholesterol (7aOHC): 0.76 $\pm$ 0.06; 7 $\beta$ -hydroxycholesterol (7bOHC): 0.68 $\pm$ 0.07; 7-ketocholesterol (7-Ketochol): 0.24 $\pm$ 0.03; cholesterol-5 $\alpha$ ,6 $\alpha$ -epoxide (a-Epoxychol): 0.21 $\pm$ 0.02; cholesterol-5 $\beta$ ,6 $\beta$ -epoxide (b-Epoxychol): 0.08 $\pm$ 0.01. Each value represents the mean  $\pm$  S.E.M. of 8-10 independent experiments.

\*p<0.05, \*\*p<0.01, \*\*\*p<0.001 versus sham; #p<0.05, ##p<0.01, ###p<0.001 versus ischemia-reperfusion (I/R).

**Figure 5:** Schematic representation of the proposed mechanism which may contribute to the cardioprotective effect of TSPO ligands.

A: In control conditions, mitochondrial cholesterol (CHOL) concentrations and oxidative stress are low and thus there is low production of oxysterols.

B: Myocardial reperfusion generates oxidative stress and increases the accumulation of cholesterol into mitochondria via the mitochondrial translocator protein (TSPO). This induces the formation of oxysterols which can promote lipid peroxidation and the increase in membrane permeability.

C: The administration of TSPO ligands inhibits the accumulation of CHOL into mitochondria and thus may prevent oxysterol formation.

Table 1: Measurement of mitochondrial parameters after myocardial ischemia-reperfusion.

Mitochondrial oxygen consumption and calcium retention capacity were expressed as nanomoles of oxygen per minute per milligram of protein, and nanomoles per milligram of protein, respectively. Each value represents the mean  $\pm$  S.E.M. of 5 independent experiments performed in triplicate.

\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus sham; # $p < 0.05$ , ## $p < 0.01$  versus ischemia-reperfusion (I/R).

	Sham	I/R	I/R + 4'-chlorodiazepam (10 mg/kg)
Oxygen consumption			
State 3	412 $\pm$ 17.4	298 $\pm$ 18.7 <sup>**</sup>	362 $\pm$ 22.3 <sup>#</sup>
State 4	75.9 $\pm$ 1.40	82.7 $\pm$ 2.60 <sup>*</sup>	79.0 $\pm$ 2.24
Respiratory control ratio	5.46 $\pm$ 0.24	3.62 $\pm$ 0.22 <sup>***</sup>	4.60 $\pm$ 0.27 <sup>#</sup>
Calcium retention capacity	92.3 $\pm$ 4.30	46.1 $\pm$ 4.50 <sup>***</sup>	70.2 $\pm$ 4.80 <sup>##</sup>

Table 2: Effect of myocardial ischemia-reperfusion on mitochondrial membrane fluidity and lipid peroxidation.

Fluidity of mitochondrial membranes was evaluated by fluorescence anisotropy of mitochondria-bound dyes 1,6-diphenyl-1,3,5-hexatriene (DPH) and hematoporphyrin IX (HP), and lipid peroxidation was assessed as generation of conjugated dienes and TBARs.

Fluidity of mitochondrial membranes was also evaluated in cholesterol overloaded mitochondria isolated from control hearts (non ischemia-reperfused).

Each value represents the mean  $\pm$  S.E.M. of 8-12 (membrane fluidity), 8-14 (conjugated dienes) and 5-6 (TBARs) independent experiments.

\*p<0.05, \*\*p<0.01 versus sham; #p<0.05, ##p<0.01 versus ischemia-reperfusion (I/R).

	Sham	I/R	I/R + 4'-chlorodiazepam (10 mg/kg)	Cholesterol overloaded mitochondria
Fluorescence anisotropy				
Lipid regions (DPH) (Arbitrary unit)	0.189 $\pm$ 0.002	0.198 $\pm$ 0.003 *	0.183 $\pm$ 0.002 ##	0.205 $\pm$ 0.004 **
Protein regions (HP) (Arbitrary unit)	0.259 $\pm$ 0.005	0.239 $\pm$ 0.005 *	0.241 $\pm$ 0.006	0.256 $\pm$ 0.005
Lipid peroxidation				
Conjugated dienes formation (Optic density)	0.250 $\pm$ 0.015	0.335 $\pm$ 0.023 **	0.248 $\pm$ 0.019 #	-
TBARs formation (nmoles/mg Pt)	1.82 $\pm$ 0.07	2.47 $\pm$ 0.12 *	1.79 $\pm$ 0.10 #	-

Figure 1

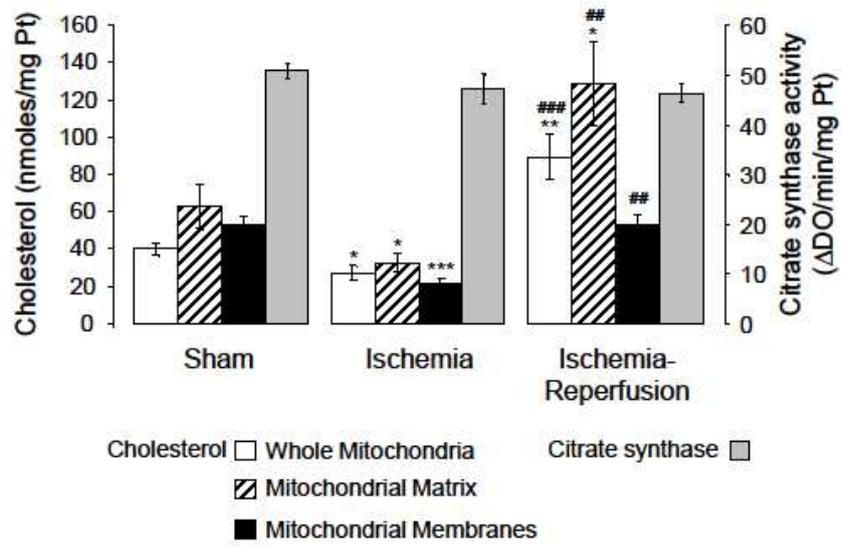


Figure 2

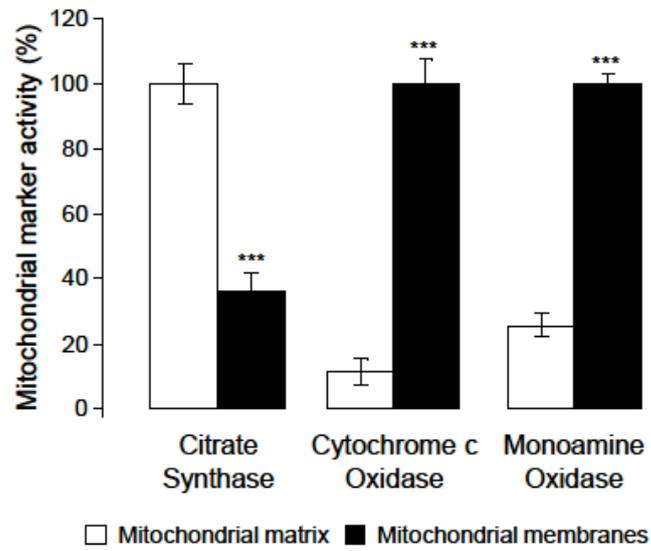


Figure 3

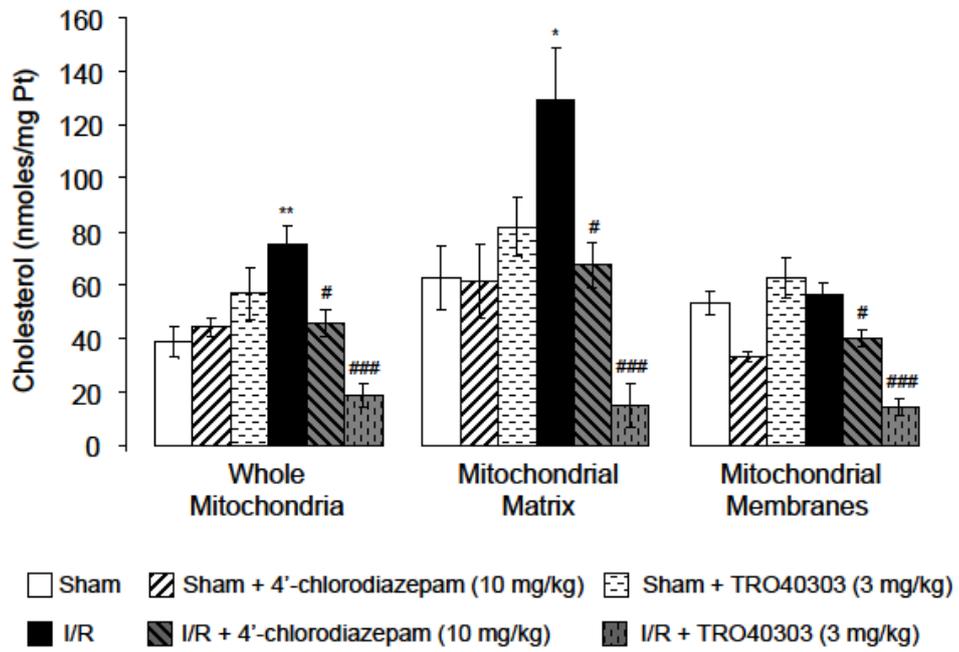


Figure 4

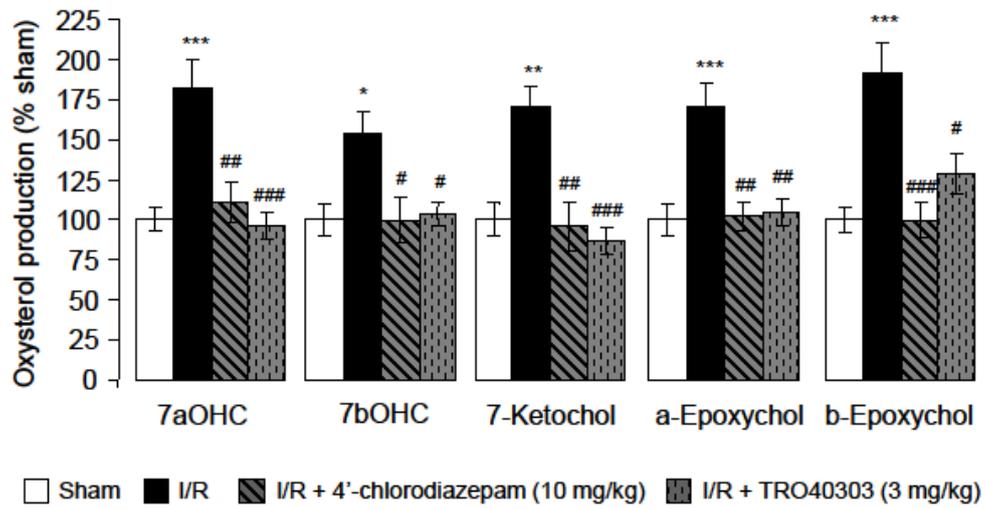


Figure 5

