Peripheral Benzodiazepine Receptor-Induced Myocardial Protection is Mediated by Inhibition of Mitochondrial Membrane Permeabilization

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ABSTRACT
Opening of the permeability transition pore (PTP) is a key event in ischemia-reperfusion injury and several ligands of the peripheral benzodiazepine receptor (PBR), a mitochondrial outer membrane protein possibly associated with PTP, have been demonstrated as potent cardioprotective agents. Here, we investigated the mechanisms by which the specific PBR ligand 4'-chlorodiazepam (CDZ) protected the myocardium against ischemia-reperfusion. In either global or regional models of myocardial ischemia-reperfusion in rats, CDZ reduced infarct size in a dose-dependent manner (e.g., 11 ± 1% of the area at risk at 10 mg/kg versus 31 ± 3% in control; p < 0.05) and to a similar extent as ischemic or diazoxide-induced preconditioning. CDZ (10 mg/kg) reduced apoptosis (terminal deoxynucleotidyl transferase dUTP nick-end labeling staining), restored mitochondrial recovery, improved oxidative phosphorylation parameters, and reduced mitochondrial membrane permeabilization with inhibition of cytochrome c and apoptosis-inducing factor releases. CDZ increased the resistance of mitochondria to Ca2+-induced PTP opening. All these cardioprotective effects of CDZ were associated with an improved stabilization of the association of Bcl-2 with the mitochondrial membrane and inhibition of the association of a cytosolic fragment of Bax, occurring during ischemia-reperfusion, with the outer mitochondrial membrane. In addition, the PTP opener atractyloside (20 μM) and the Bcl-2 inhibitor ethyl-2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate (HA14-1) (20 μM) abrogated CDZ-induced reduction of infarct size. These results demonstrate that PBR occupancy by CDZ renders the heart more resistant to ischemia-reperfusion injury by limiting mitochondrial membrane permeabilization. This is due to a reorganization of the balance between pro- and anti-apoptotic proteins of the Bcl-2 family proteins at the level of mitochondrial membranes.

It is now well established that during myocardial ischemia-reperfusion, pathological signals converge to the mitochondria and induce its membrane permeabilization, a phenomenon that ultimately leads to cell death (Gottlieb, 2003; Juhaszova et al., 2004; Lundberg and Szwed, 2004). Two mechanisms have been described to explain mitochondrial membrane permeabilization (Green, 2005). The first mechanism concerns permeabilization of the outer membrane with alteration of the inner membrane, which can be facilitated by the proapoptotic members of the Bcl-2 family proteins. The second mechanism involves both the inner and the outer membranes, and it corresponds to the opening of the permeability transition pore (PTP), a multiprotein structure whose opening leads to mitochondrial swelling and subsequent release of apoptogenic factors (Zoratti and Szabo, 1995).

Through mitochondrial membrane permeabilization, ischemia-reperfusion (I/R) induces the release of cell death effectors and ultimately results in the loss of mitochondrial functions that are fundamental for cell survival. Therefore, every strategy able to limit mitochondrial membrane permeabilization...
zation is potentially cardioprotective, and PTP is a highly relevant target for this purpose. One of the proteins that might regulate PTP is the peripheral benzodiazepine receptor (PBR), an 18-kDa protein primarily located on the outer mitochondrial membrane and associated with the voltage-dependent anion channel (McEnery, 1992). However, the physiological role of PBR remains unclear, except in specific tissues involved in steroidogenesis (Lacapère and Papadopoulos, 2003) where PBR mediates the transport of cholesterol from the outer to the inner mitochondrial membrane and promotes pregnenolone synthesis. PBR is also present in nonsteroidogenic tissues, especially in the heart where its function is unknown. Several reports suggest that PBR ligands might modulate apoptotic responses (Bono et al., 1999) and play an antiapoptotic role in oxidative stress conditions such as ischemia-reperfusion (Carayon et al., 1996). In agreement with this role, Leducq et al. (2003) showed that the irreversible PBR ligand SSR180575 prevented the cellular damages against oxidative stress and cardiac injuries induced by ischemia-reperfusion in rodents, but the mechanism involved in these effects remains unclear.

Therefore, the primary goal of the present study was to determine the mechanism by which PBR protected the myocardium against ischemia-reperfusion. For this purpose, we investigated the effects of the specific PBR benzodiazepine ligand Ro5-4864, generically known as 4'-chlorodiazepam (CDZ), in two models of myocardial ischemia-reperfusion in rat. CDZ binds with high affinity to the PBR from rodent species and with low affinity to GABA receptors (Marangos et al., 1982; Schoemaker et al., 1983). We used infant size as the endpoint of injury to ascertain that PBR occupancy protects the myocardium against the consequences of lethal ischemia-reperfusion injury. Then, we determined whether some main mitochondrial functions (e.g., respiration and oxidative phosphorylation) and its membrane permeabilization were modified by PBR occupancy during ischemia-reperfusion. More precisely, we examined whether PBR occupancy affected PTP opening and modified the interaction of Bcl-2 family proteins with the mitochondrial membrane.

Materials and Methods

Animals. All animal procedures used in this study were in strict accordance with the European Community Council Directive (86/864/ECC) and recommendations of the French Ministère de l’Agriculture.

Wistar rats, weighing 250 to 280 g, 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), and they were acclimatized to the animal room before being used. They received standard pelleted rat diet and water ad libitum.

Isolated Perfused Rat Heart Submitted to Global Ischemia and Reperfusion. Rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and then intubated and ventilated with room air using a respirator (model 683; Harvard Apparatus Inc., Holliston, MA). 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 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 (CAO), and releasing the ends of the suture initiated reperfusion. In all groups of rats, the coronary artery was occluded during 35 min, and released for reperfusion, except in the so-called sham group in which the surgical procedure was identical to others, but the coronary artery was not occluded. In the other groups of treated rats, vehicle or increasing doses of CDZ (0.5–10 mg/kg) were administered separately as a 5-min infusion through the jugular vein 10 min before CAO. Finally, ischemic preconditioning using three periods of 5 min CAO/5-min reperfusion was made before the onset of the 35-min CAO in another group of rats. At the end of the experiments, the chest was closed in layers and the pneumothorax was evacuated.

Rats were allowed to recover for 24 h before measurement of infarct size. In experiments used for determination of mitochondrial functions (e.g., respiratory rate, oxidative phosphorylation, PTP opening) and Western blot analysis or assessment of myocardial apoptosis, rats were sacrificed after 1 and 3 h of reperfusion, respectively.

Infarct Size Determination. After completion of reperfusion, rats received heparin (1000 IU/kg), and then they were reanesthetized with sodium pentobarbital (60 mg/kg). After thoracotomy, the coronary artery was reooccluded on the same place as used previously to induce myocardial infarction 24 h before. Potassium chloride was used to induce cardiac arrest, and the hearts were excised. The ascending aorta was cannulated and perfused retrogradely (Langendorff apparatus; 100 mm Hg) with saline followed by 5% Evans blue dye. The area at risk (AAR) was the region of the left ventricle not colored in blue. Infarct size was determined as the percentage of AAR. Total ventricle (global ischemia) in the isolated heart studies or the left ventricle (regional ischemia) in in vivo studies was cut from apex to base into six slices that were weighed and incubated with 1% triphenyltetrazolium chloride (Sigma Chemical, Poole, Dorset, UK) in a pH 7.4 buffer during 20 min at 37°C. Slices were fixed overnight in 10% formaldehyde and then photographed with a digital camera mounted on a stereomicroscope. Using a computerized planimetric Image Analyser (Secon Camera, Frederic, CA), the total and the infarcted zones were quantified. AAR was expressed as a percentage of the left ventricle weight, and infarct size was expressed as heart rate, maximal first derivative of ventricular pressure (dP/dt), and coronary flow (CF) were recorded every 10 s using emka software (emka Technologies, Paris, France).

All investigated drugs were administered as one concentration in a separate isolated heart preparation. In a first set of experiments, the vehicle dimethyl formamide as control, CDZ (1 μM), or diazoxide (30 μM) was administered as a 15-min infusion, 15 min before the 20-min global ischemia. For comparison, ischemic preconditioning (IPC) was made with three periods of 5-min global ischemia/5-min reperfusion 10 min before the 20-min global ischemia. In a second set of experiments, vehicle, CDZ at increasing concentrations (0.1 nM, 10 nM, and 1 μM), the isoquinoline PBR ligand PK-11195 (1 μM), or CDZ associated with PK-11195 (both 1 μM) was administered as a constant infusion from the onset until the end of reperfusion. In addition, the PTP opener atracylyside (20 μM) and the selective Bcl-2 inhibitor HA14-1 (20 μM; Wang et al., 2000) were administered in this second set of experiments 5 and 30 min before ischemia induction, respectively, and continued as a constant infusion rate from the onset until the end of reperfusion in the absence or in the presence of 1 μM CDZ given at reperfusion. The concentrations of CDZ used were chosen according to the Kd value of the drug for PBR in isolated cardiac mitochondria (Kd ≈ 10 nM in our experimental conditions; data not shown).

In Vivo Model of Coronary Occlusion-Reperfusion. Rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and then intubated and ventilated with room air using a respirator (model 683; Harvard Apparatus Inc., Holliston, MA). 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 (CAO), and releasing the ends of the suture initiated reperfusion. In all groups of rats, the coronary artery was occluded during 35 min, and released for reperfusion, except in the so-called sham group in which the surgical procedure was identical to others, but the coronary artery was not occluded. In the other groups of treated rats, vehicle or increasing doses of CDZ (0.5–10 mg/kg) were administered separately as a 5-min infusion through the jugular vein 10 min before CAO. Finally, ischemic preconditioning using three periods of 5 min CAO/5-min reperfusion was made before the onset of the 35-min CAO in another group of rats. At the end of the experiments, the chest was closed in layers and the pneumothorax was evacuated. Rats were allowed to recover for 24 h before measurement of infarct size. In experiments used for determination of mitochondrial functions (e.g., respiratory rate, oxidative phosphorylation, PTP opening) and Western blot analysis or assessment of myocardial apoptosis, rats were sacrificed after 1 and 3 h of reperfusion, respectively.

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a percentage of AAR (or percentage of the ventricular weight in global ischemia).

**Isolation of Mitochondrial and Cytosolic Fractions.** AAR from ischemic myocardium were minced and homogenized with an Ultra-Turrax T25 (set at position 5; IKA-Werke GmbH & Co. KG, Staufen, Germany) for 3 in a buffer containing 220 mM mannitol, 70 mM sucrose, 10 mM HEPES, and 1 mM EGTA, pH 7.4, at 4°C. This was followed by five down-passes in a Potter-Elvehjem glass homogenizer (motor-driven Teflon pestle set at 1500 rpm) in a final volume of 5 ml. Cardiac homogenates were centrifuged at 1000g for 5 min at 4°C, and the supernatants were centrifuged at 10,000g for 10 min at 4°C. Mitochondrial pellets were resuspended in the homogenization buffer and centrifuged one more time at 10,000g for 10 min. The final mitochondrial pellets were resuspended in the homogenization buffer without EGTA to obtain a protein concentration of about 15 mg/ml. The 10,000g supernatants were centrifuged at 40,000g for 30 min. The supernatants were considered as the particulate-free cytosolic fractions. Protein concentrations were determined by the method of Lowry et al. (1951).

**Evaluation of Mitochondrial Oxygen Consumption and PTP Opening in Isolated Mitochondria.** Oxygen consumption was measured at 37°C with a Clark-type electrode. Mitochondria (0.4 mg/ml) were incubated in a respiration buffer containing 100 mM KCl, 50 mM sucrose, 10 mM HEPES, and 5 mM KH$_2$PO$_4$, pH 7.4, at 37°C. Respiration was initiated by addition of 10 mM pyruvate/malate (state 2 respiration rate). After 1 min, ATP synthesis was induced by addition of 500 μM ADP (state 3 respiration rate). Upon depletion of ADP, the rate of state 4 respiration was measured, and the respiratory control ratio (state 3/state 4) and the ADP/O value were calculated.

In mitochondria isolated from hearts subjected to coronary occlusion-reperfusion, PTP opening was assessed by monitoring mitochondrial Ca$^{2+}$ retention. When maximal Ca$^{2+}$ release was reached, this equilibrium was disrupted, and Ca$^{2+}$ was released. The concentration of Ca$^{2+}$ in the extramitochondrial medium was monitored by means of a PerkinElmer LS 50B spectrofluorimeter. The Ca$^{2+}$ signal was calibrated by addition to the medium of known Ca$^{2+}$ amounts.

The direct effect of CDZ on PTP opening was studied on isolated mitochondria prepared from control hearts by analyzing mitochondrial swelling as described previously (Elimadi et al., 2003). Mitochondrial swelling was assessed by measuring the change in absorbance of the suspension at 540 nm (A$_{540}$) by using a model UV-3000 spectrophotometer (Hitachi, Tokyo, Japan). Experiments were carried out at 37°C in 1.5 ml of respiration buffer with addition of 10 mM pyruvate/malate. Mitochondria (0.4 mg/ml) were incubated for 1 min in the respiration buffer, and swelling was induced by addition of 200 μM Ca$^{2+}$ or increasing concentrations of CDZ.

**Western Blot Analysis.** Samples of cytosolic proteins (5 μg for cytochrome c, 15 μg for apoptosis-inducing factor (AIF), and 30 μg for Bax and Bcl-2) and mitochondrial extracts (2 μg for cytochrome c and AIF and 40 μg for Bax, Bcl-2, and PBR) were boiled at 95°C in a buffer containing 20% sucrose, 2.4% SDS, 5% β-mercaptoethanol, and 5% bromphenol blue. They were subjected to electrophoresis on 4 to 15% (Cytochrome c, Bcl-2, PBR, and Bax) and 4 to 10% (AIF) gradient SDS-polyacrylamide gel electrophoresis gels and then transferred on polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat dry milk in a Tris buffer (10 mM Tris and 100 mM NaCl, pH 7.5) containing 0.05% Tween 20 overnight at 4°C. Subsequently, membranes were exposed for 1 h to either mouse cytochrome c antibody (1:1000; BD Biosciences, San Jose, CA), goat polyclonal anti-AIF antibody (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal anti-Bax and anti-Bcl-2 antibodies (1:500; Santa Cruz Biotechnology), rabbit polyclonal anti-P53 antibody (1:2500), anti-actin-biotin conjugate antibody (1:2000; Santa Cruz Biotechnology, Inc.), goat anti-rabbit (Santa Cruz Biotechnology, Inc.), donkey anti-goat (Santa Cruz Biotechnology, Inc.), or anti-streptavidin horseradish peroxidase as a secondary antibody at 1:5000, blots were revealed by enhanced chemiluminescence reaction (ECL kit; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and exposed to X-rays film (GE Healthcare). Band intensities were analyzed by densitometry using NIH Image J software (http://rsb.info.nih.gov/ij/).

**Binding Experiments.** Mitochondria were broken by freezing and thawing. Then, they were incubated (0.05 mg/ml protein) in a 25 mM phosphate buffer, pH 7.4, with increasing concentrations (0.2–20 nM) of [3H]PK-11195 (83.5 Ci/mmol) for 60 min at 4°C in a total volume of 250 μl. Specific binding was defined as the difference between total binding and binding in the presence of 100 μM flunitrazepam.

At the end of the incubation period, bound and free ligands were separated by rapid filtration through Whatman GF/B glass fiber filters (Whatman, Maidstone, UK) (presoaked in 0.1% polyethyleneimine). Each filter was washed twice with an additional 5 ml of ice-cold 25 mM phosphate buffer and counted in a Packard 1600 TR liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Boston, MA) with an efficiency of 45%. Binding parameters (maximal density of sites, B$_{max}$ and dissociation constant, K$_D$) were analyzed by means of nonlinear regression with a commercially available software (MicroPharm, version 1; Institut National de la Santé et de la Recherche Médicale, Paris, France) as described previously (Morin et al., 1998).

**Assessment of Myocardial Apoptosis.** After 3 h of reperfusion, the hearts were excised, fixed with formalin, and embedded in paraffin. Apoptosis was quantified by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) technique (Apoptosis detection kit ApoTag; Chemicon International, Temecula, CA). Paraffin-fixed sections (4 μm) of frozen nonischemic and ischemic left ventricles were treated with proteinase K (20 μg/ml) and then with peroxidase in the presence of conjugated digoxigenin and terminal deoxynucleotidyl transferase enzyme at 37°C. Positive controls were achieved by the treatment of DNase I (1 μg/ml). Nuclei of cardiomyocytes labeled with 10% hematoxylin were defined as TUNEL-positive. For each section, five randomly separate fields were examined with an Olympus microscope (Olympus, Tokyo, Japan) using a ×40 objective. The mean percentage of TUNEL-positive cardiomyocytes was expressed as the number of TUNEL-positive cells relative to the total number of cardiomyocytes.

**Statistical Analysis.** The data are reported as mean ± S.E.M. Comparisons between groups were performed using a one-way analysis of variance followed by Scheffe’s test. Significance was accepted when p < 0.05.

**Results**

**4-Chlorodiazepam Reduced Infarct Size in Isolated Myocardium.** As shown in Fig. 1A, CDZ (1 μM) administered before global ischemia reduced infarct size (6 ± 4 versus 18 ± 2% in control ischemia; p < 0.05) to a similar extent as ischemic and pharmacological (30 μM diazoxide) preconditioning (5 ± 2 and 8 ± 2%, respectively). As shown in Fig. 1B, significant reductions in infarct size were also observed.
when CDZ (from 10 nM to 1 μM) as well as the other high-affinity PBR ligand, PK-11195 (1 μM), was administered at reperfusion. However, the association of CDZ and PK-11195 (both 1 μM) did not afford a higher protection than that observed when each drug was administered separately. As shown in Fig. 1C, the ATP opener atractyloside (20 μM) and the selective Bcl-2 inhibitor HA14-1 (20 μM) abolished the reduction of infarct size induced by 1 μM CDZ, whereas each drug administered separately did not affect infarct size. As shown in Table 1, baseline parameters related to cardiac function and coronary flow were not different in experimental and control I/R groups. 

**TABLE 1**

<table>
<thead>
<tr>
<th>Functional data measured before and during global I/R in control and treated isolated rat hearts</th>
</tr>
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<tbody>
<tr>
<td><strong>Control I/R</strong> (n = 9)</td>
</tr>
<tr>
<td><strong>Baseline</strong></td>
</tr>
<tr>
<td>LVDP (mm Hg)</td>
</tr>
<tr>
<td>dP/dt_max (mm Hg/s)</td>
</tr>
<tr>
<td>HR (beats/min)</td>
</tr>
<tr>
<td>CF (ml/min)</td>
</tr>
<tr>
<td><strong>20-min ischemia</strong></td>
</tr>
<tr>
<td>LVDP (mm Hg)</td>
</tr>
<tr>
<td>dP/dt_max (mm Hg/s)</td>
</tr>
<tr>
<td>HR (beats/min)</td>
</tr>
<tr>
<td>CF (ml/min)</td>
</tr>
<tr>
<td><strong>End of reperfusion</strong></td>
</tr>
<tr>
<td>LVDP (mm Hg)</td>
</tr>
<tr>
<td>dP/dt_max (mm Hg/s)</td>
</tr>
<tr>
<td>HR (beats/min)</td>
</tr>
<tr>
<td>CF (ml/min)</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. corresponding control I/R values.
imental groups before the ischemic period. CDZ improved cardiac function (assessed by the left ventricular developed pressure) when CDZ was administered before ischemia or at reperfusion (42 ± 6 and 52 ± 4 mm Hg, respectively, versus 30 ± 2 mm Hg in control ischemia). A similar improvement in cardiac function was also observed with ischemic preconditioning, diazoxide and PK-11195.

4'-Chlorodiazepam Reduced Infarct Size in Vivo. The AAR in infarcted rats was not significantly different between groups, ranging from 34 ± 9 to 39 ± 3%. As shown in Fig. 2, myocardial infarct size was 31 ± 3% AAR (versus 3 ± 1% in corresponding sham group) in the control group of rats receiving the vehicle of CDZ as treatment before 35-min regional ischemia-24-h reperfusion. CDZ reduced infarct size at doses of 5 and 10 mg/kg (16 ± 2 and 11 ± 1%, respectively; \( p < 0.05 \)), with the reduction observed at CDZ 10 mg/kg being of a similar extent as that induced by ischemic preconditioning in the same experimental conditions (11 ± 3%).

4'-Chlorodiazepam Reduced Apoptosis. As shown in Fig. 3, TUNEL-positive cells were observed after 3-h reperfusion compared with corresponding sham-operated rats and CDZ (10 mg/kg) decreased their number compared with corresponding rats in which the same ischemia-reperfusion was performed after vehicle administration (6 ± 2 versus 22 ± 9%; \( p < 0.05 \)).

4'-Chlorodiazepam Improved Oxidative Phosphorylation and Limited Mitochondrial Membrane Permeabilization. As shown in Table 2, 35-min ischemia followed by 1-h reperfusion performed in control rats decreased the yield of mitochondria assessed by measuring mitochondrial proteins per gram of wet myocardial weight in the mitochondrial pellet. This yield corresponded to 74% of that measured in corresponding sham-operated rats. Administration of 10 mg/kg CDZ before ischemia restored the yield (98% of corresponding sham-operated rats).

As also shown in Table 2, ischemia-reperfusion decreased the respiratory control ratio. This was related to the decrease in state 3 respiratory rate, because corresponding states 2 and 4 were not modified, indicating that the integrity of the mitochondrial inner membrane of isolated mitochondria was not affected. This was confirmed by the lack of change in the respiratory rate following addition of the specific PTP inhibitor cyclosporin A in the incubation medium, indicating that PTP opening was not involved (data not shown). In fact, ischemia-reperfusion induced permeabilization of the mitochondrial membrane because cytochrome c and AIF, two proteins located in the intermembrane space, occurred by that time in the cytosolic extracts isolated from ischemic and reperfused myocardium (Fig. 4). Simultaneously, cytochrome c and AIF decreased in the mitochondrial fraction, indicating that the appearance of these proteins in the cytosol was due to permeabilization of the mitochondrial membranes. Importantly, this phenomenon could only be observed when low quantities of mitochondria (i.e., 0.2 and 2 μg) were used for Western blot analysis of cytochrome c and AIF, respectively. This indicates that the quantity of proteins released in the cytosol probably represented a small fraction of the total mitochondrial pool. As also shown in Fig. 4, CDZ inhibited the release of these proteins as demonstrated by the simultaneous decrease in the cytosolic extracts and restoration of the mitochondrial pools. This protection of CDZ against ischemia-reperfusion-induced mitochondrial membrane permeabilization was associated with an improvement in oxidative phosphorylation (CDZ limited the decrease in respiratory...
control ratio) by increasing the state 3 respiration rate and improvement in the capacity of mitochondria to produce ATP as evidenced by restoration of the ADP/O value (Table 2). It should be pointed out that CDZ did not exhibit any direct effect on mitochondrial respiration when it was examined on control isolated cardiac mitochondria (Table 3).

**Table 2**: Measurement of mitochondrial parameters

Mitochondrial yields, O₂ consumption, and Ca²⁺ concentration-induced PTP opening were expressed as milligrams per gram of wet weight, nanomoles of O₂ per minute per milligram of proteins, and micromoles per milligram of proteins, respectively. Each value is the mean ± S.E.M. of 13 independent mitochondrial preparations.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>I/R</th>
<th>I/R + 10 mg/kg CDZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial yield</td>
<td>8.62 ± 0.68</td>
<td>6.43 ± 0.36*</td>
<td>8.48 ± 0.80</td>
</tr>
<tr>
<td>O₂ consumption</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>State 2 respiration</td>
<td>162 ± 3</td>
<td>148 ± 3</td>
<td>160 ± 3</td>
</tr>
<tr>
<td>State 3 respiration</td>
<td>702 ± 6</td>
<td>472 ± 12*</td>
<td>593 ± 10#</td>
</tr>
<tr>
<td>State 4 respiration</td>
<td>229 ± 3</td>
<td>236 ± 4</td>
<td>253 ± 4</td>
</tr>
<tr>
<td>Oxidative phosphorylation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory control</td>
<td>3.75 ± 0.17</td>
<td>2.45 ± 0.26*</td>
<td>3.11 ± 0.24#</td>
</tr>
<tr>
<td>ADP/O</td>
<td>2.52 ± 0.02</td>
<td>1.72 ± 0.06*</td>
<td>2.12 ± 0.06#</td>
</tr>
<tr>
<td>Ca²⁺-induced PTP opening</td>
<td>659 ± 48</td>
<td>413 ± 28*</td>
<td>559 ± 38#</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. sham.
# p < 0.05 vs. I/R.

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control ratio) by increasing the state 3 respiration rate and improvement in the capacity of mitochondria to produce ATP as evidenced by restoration of the ADP/O value (Table 2). It should be pointed out that CDZ did not exhibit any direct effect on mitochondrial respiration when it was examined on control isolated cardiac mitochondria (Table 3).

**4’-Chlorodiazepam Modulated the Association of Proteins of the Bcl-2 Family with Mitochondrial Membranes.** Table 2 shows that the amount of Ca²⁺ necessary to trigger PTP opening reached 659 ± 48 nmol Ca²⁺ per milligram of mitochondrial proteins in sham-operated rats. Ischemia-reperfusion resulted in a significant reduction of Ca²⁺ load necessary to induce PTP opening, whereas CDZ administration limited this decrease. It should be noticed that CDZ did not inhibit PTP opening assessed by mitochondrial swelling and triggered by high Ca²⁺ in mitochondria isolated from a control myocardium (Fig. 5A). Moreover, at high concentrations CDZ was able to induce PTP opening (Fig. 5B).

As shown in Fig. 6, we found also evidence that CDZ redistributed the Bcl-2 family proteins, because the release of Bcl-2 from mitochondria during ischemia-reperfusion was prevented by CDZ, which stabilized its association with the mitochondrial membrane. Ischemia-reperfusion also modified the cellular location of Bax immunoreactivity. When cytosolic and mitochondrial extracts were probed with an antibody specific of the N terminus of Bax, the 21-kDa isoform of Bax was detected in the two fractions, and its level did not vary significantly according to the experimental protocol (Fig. 6), although a slight but significant decrease was systematically observed in the cytosolic fraction after ischemia-reperfusion. The most characteristic phenomenon was the appearance of a Bax-reactive band around 10 kDa in the cytosolic and mitochondrial fractions after ischemia-reperfusion, and CDZ significantly reduced this association. This band was not present in both cytosolic and mitochondrial fractions isolated from myocardium of sham-operated rats.

**Ischemia-Reperfusion Did Not Alter the Expression of the PBR Receptor.** Several data have suggested that the absence or the decreased level of PBR produced breaks in the outer mitochondrial membranes (Papadopoulos et al., 1999). Thus, we compared the level of mitochondrial PBR protein before and after ischemia-reperfusion. PBR was either la-

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**Fig. 4.** CDZ limited AIF and cytochrome c (cyt c) releases from mitochondria during I/R. Neither changes in actin as cytosolic protein nor cytochrome oxidase subunit IV (Cox IV) as mitochondrial protein were observed, indicating equal protein loading. Western blots shown are representative of five such experiments. * p < 0.05 versus sham. # p < 0.05 versus I/R.
TABLE 3
Effect of CDZ on mitochondrial respiration of control isolated cardiac mitochondria

Mitochondria (0.4 mg/ml) were incubated at 37°C in the respiration buffer containing 10 mM pyruvate/malate in the presence or in the absence of increasing concentrations of CDZ. State 3 respiration was induced by addition of 500 μM ADP. Data were expressed in percentage of control values. Control values were 379 ± 64 nmol O2/min/mg proteins, 88 ± 11 nmol O2/min/mg proteins, and 4.54 ± 0.55 for state 3 respiration rate, state 4 respiration rate, and respiratory control ratio, respectively. Each value is the mean ± S.E.M. of five independent mitochondrial preparations.

<table>
<thead>
<tr>
<th>Control Ratio</th>
<th>State 3</th>
<th>State 4</th>
<th>Respiratory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 6.8</td>
<td>100.0 ± 5.1</td>
<td>100.0 ± 4.9</td>
</tr>
<tr>
<td>CDZ 10⁻¹⁰ M</td>
<td>100.0 ± 8.1</td>
<td>92.1 ± 9.1</td>
<td>104.4 ± 3.3</td>
</tr>
<tr>
<td>CDZ 10⁻⁹ M</td>
<td>95.8 ± 10.0</td>
<td>91.4 ± 7.7</td>
<td>107.3 ± 3.5</td>
</tr>
<tr>
<td>CDZ 10⁻⁸ M</td>
<td>94.5 ± 6.8</td>
<td>92.6 ± 6.5</td>
<td>106.2 ± 6.8</td>
</tr>
<tr>
<td>CDZ 10⁻⁷ M</td>
<td>106.3 ± 6.7</td>
<td>98.9 ± 7.0</td>
<td>103.1 ± 4.2</td>
</tr>
<tr>
<td>CDZ 10⁻⁶ M</td>
<td>106.0 ± 7.0</td>
<td>103.2 ± 6.6</td>
<td>98.9 ± 3.6</td>
</tr>
<tr>
<td>CDZ 10⁻⁵ M</td>
<td>94.0 ± 8.2</td>
<td>102.2 ± 8.6</td>
<td>92.9 ± 4.9</td>
</tr>
<tr>
<td>CDZ 10⁻⁴ M</td>
<td>86.1 ± 2.3*</td>
<td>123.0 ± 5.8*</td>
<td>70.6 ± 4.8*</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. respective control values.

The present data demonstrate that CDZ, a well known ligand of PBR, reduced myocardial infarct size when administered either before or after ischemia-reperfusion and that it inhibited mitochondrial membrane permeabilization, a property associated with a significant reduction in apoptosis and a redistribution of the Bcl-2 family proteins at the mitochondrial level.

Identical reduction in infarct size was observed with the isooxazine carboxamide PK-11195, another well known PBR ligand. Based on thermodynamic features (Le Fur et al., 1983) and opposite interactions on the proapoptotic effect of tumor necrosis factor-α (Bono et al., 1999), these ligands were classified as agonist and antagonist, respectively. However, the functional significance of such a classification remains ambiguous and even contradictory, ranging from stimulatory to inhibitory responses in several physiological paradigms (Mukhin et al., 1989; Papadopoulos et al., 1990), probably reflecting a tissue specificity of PBR function. This is what we observed here in isolated perfused myocardium following global ischemia-reperfusion where these two PBR ligands displayed the same protective effects when they were associated as that obtained when they were administered separately.

Regardless of the experimental model used, either global ischemia in isolated heart or regional ischemia in anesthetized rat, CDZ significantly reduced infarct size with a magnitude quite similar to that obtained with ischemic and pharmacological preconditioning. Interestingly, CDZ was as effective when it was administered before or immediately after ischemia, confirming that targeting PBR protects the myocardium against the deleterious effects induced by both ischemic and reperfusion insults.

Among the mechanisms involved in the induction of reperfusion injuries, permeabilization of the mitochondrial membrane leading to the release of protease and nuclease activators and to bioenergetic failure is a crucial event. Pharmacological strategies developed to inhibit this phenomenon have demonstrated their cardioprotective potencies (Mattson and Kroemer, 2003). Consistent with PBR located on the outer mitochondrial membrane in a close physical association with components of PTP, we demonstrate here, for the first time to our knowledge, that the mechanism of cardioprotection induced by CDZ at the level of PBR is linked to the limitation of mitochondrial membrane permeabilization. Indeed, CDZ limits the ischemia-reperfusion-induced release of cytochrome c and AIF, two proapoptotic proteins located in the intermembrane mitochondrial space. This permeabilization process seems to involve primarily the outer membrane of mitochondria, because the integrity of the inner membrane issued from isolated perfused hearts was not affected by ischemia-reperfusion as attested by the stability of state 2 and state 4 respiratory rates. This effect was associated with an improvement in mitochondrial respiration and oxidative phosphorylation as CDZ increased state 3 respiratory, respiratory control ratio, and ADP/O ratio values. The restoration of respiratory function by CDZ could be the consequence of the prevention of mitochondrial cytochrome c release and thus to the better availability of cytochrome c for the electron transfer chain. It might also result from a decrease in free radicals overproduction associated with prolonged ischemia-reperfusion, which has been involved in inhibition of oxidative phosphorylation (Solaini and Harris, 2005). Indeed, mitochondrial PBR was shown to promote

Fig. 5. Effect of CDZ on mitochondrial swelling. A, CDZ did not inhibit Ca²⁺-induced mitochondrial swelling. Mitochondria (0.4 mg/ml) were incubated for 2 min with CDZ, and swelling was initiated by the addition of 200 μM Ca²⁺ and monitored for 10 min. B, high concentrations of CDZ induced mitochondrial swelling. Data are presented as the mean of five independent experiments. CαA, cyclosporine A. *p < 0.05 versus control. #, p < 0.05 versus 200 μM Ca²⁺. §, p < 0.05 versus 250 μM CDZ.
antioxidant properties by an as yet unidentified pathway (Carayon et al., 1996).

We also demonstrate here that mitochondria isolated after treatment with CDZ displayed an increased resistance to Ca$^{2+}$ loading, indicating that CDZ delayed Ca$^{2+}$-induced PTP opening. This result can explain the improvement of the yield of mitochondria isolated from ischemia-reperfused myocardium caused by CDZ. Indeed, prolonged PTP opening is known to induce massive swelling of mitochondria, leading to membrane rupture and thus to lower yields of intact mitochondria after reperfusion. Such an improvement of yield was already observed with the specific PTP inhibitor cyclosporin A (Griffiths and Halestrap, 1995). Although PBR is a component of PTP (Zoratti and Szabo, 1995), it is unlikely that this effect was due to a direct inhibition of the drug on PTP, because our swelling experiments demonstrated that CDZ did not block PTP opening on isolated mitochondria as it is observed with cyclosporin A and even paradoxically induced PTP opening at high concentrations. These data confirm previous results (Chelli et al., 2001; Li et al., 2007), and they can explain the ability of these ligands to overcome apoptosis resistance conferred by Bcl-2 in tumor cells (Decaudin et al., 2002). However, these effects were observed at higher concentrations of PBR ligands ($\approx 100 \mu$M) than those used in the present study and at several orders of magnitude higher than the concentrations required to saturate the receptor, suggesting that these proapoptotic effects could be unrelated to PBR expression and to PBR ligand binding (Kletsas et al., 2004; Gonzalez-Polo et al., 2005).

Conversely, the data from the present study strongly suggest that PBR occupancy increased the resistance of the mitochondria to PTP opening during ischemia-reperfusion. This finding is supported by the present observation that the effect of CDZ on PTP opening was abolished by the PTP opener atracyloside.

This confirms that PTP is an important target for cardioprotection as observed with specific inhibitors (Griffiths and Halestrap, 1993; Argaud et al., 2005) and its role in pre- and postconditioning (Argaud et al., 2005). However, the mechanism by which PBR ligands modulate PTP opening is probably different, because they are not specific inhibitors of PTP, and they do not activate the cascade of prosurvival kinases, which provides powerful cardioprotection against myocardial ischemia-reperfusion injury (Hausenloy et al., 2005). In contrast, we think that CDZ promotes a reorganization of the Bcl-2 family proteins at the level of the outer mitochondrial membrane. It is well established that mitochondrial membrane permeabilization is under the control of these proapoptotic members (e.g., Bax, Bak, or the BH3-only subfamily proteins), which facilitate membrane permeabilization and promote the release of cytochrome $c$ and other intermembrane space components, such as AIF during ischemia-reperfusion. The mechanism promoting this increase in permeability is still unresolved, but different hypothesis have been proposed (Zamzami and Kroemer, 2003). One hypothesis is that these proapoptotic proteins are located in the cytosol, and during ischemia-reperfusion they translocate to the mitochondria where they incorporate into the membrane to form either a permeable pore and/or they interact with other channels, such as voltage-dependent anion channel or adenine nucleotide translocase. Conversely, the antiapoptotic members Bcl-2 and Bcl-xL are mainly located on the mitochondrial outer membrane where they antagonize the proapoptotic effect of Bax and Bak (Letali et al., 2002). The ultimate resistance or sensitivity of cells to apoptotic stimuli such as hypoxia, oxidative stress, or Ca$^{2+}$ overload encoun-

![Fig. 6](https://example.com/image.png)

**Fig. 6.** CDZ altered the distribution of mitochondrial and cytosolic fractions of Bax and Bcl-2 during I/R. Western blots shown are representative of five such experiments. *, $p < 0.05$ versus sham. #, $p < 0.05$ versus I/R.
cytochrome c release elicited by Bax in neural cells lines (Fiskum et al., 2000).

How a PBR ligand such as CDZ modifies the balance between pro- and antiapoptotic proteins of the Bcl-2 family proteins at the level of mitochondrial membrane will need further investigation. However, the present study further demonstrates that PBR is an interesting target to understand the pathophysiological mechanisms involved during myocardial ischemia-reperfusion and confirms that anti-apoptotic interventions targeting Bcl-2 family proteins provide opportunities for anti-ischemic therapies at the mitochondrial level (Mattson and Kroemer, 2003).

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References


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