Role of Peripheral Benzodiazepine Receptors in Mitochondrial, Cellular, and Cardiac Damage Induced by Oxidative Stress and Ischemia-Reperfusion

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ABSTRACT
Mitochondrial dysfunction has been identified as a possible early event in ischemia-reperfusion damage. The peripheral benzodiazepine receptor, a mitochondrial inner membrane protein, has already been proposed to play a role in mitochondrial regulation, although its exact function remains unclear. The aim of this work was to determine the role of peripheral benzodiazepine receptor ligand, 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1H-1,4-benzodiazepine-2-one (SSR180575). To characterize and link the mitochondrial, cellular, and cardiac consequences of ischemia-reperfusion, we examined the effects of SSR180575 in several in vitro and in vivo models of oxidative stress. Hydrogen peroxide decreased mitochondrial membrane potential, reduced oxidative phosphorylation capacities, and caused cytochrome c release, caspase 3 activation, and DNA fragmentation. SSR180575 (100 nM–1 µM) prevented all these effects. In perfused rat hearts, SSR180575 administered in vitro (100 nM–1 µM) or by oral pretreatment (3–30 mg/kg) greatly reduced the contractile dysfunction associated with ischemia-reperfusion. Furthermore, in anesthetized rats, SSR180575 (3–30 mg/kg p.o.) produced significant reductions in infarct size after coronary artery occlusion/reperfusion. In conclusion, we have demonstrated that peripheral benzodiazepine receptor play a major role in the regulation of cardiac ischemia-reperfusion injury and that SSR180575, a novel peripheral benzodiazepine receptor ligand, is of potential interest in these indications.

After acute coronary artery thrombosis, salvage of the ischemic heart usually requires restoration of coronary perfusion by performing routine interventions such as coronary artery bypass surgery, percutaneous transluminal coronary angioplasty, or thrombolysis. Despite recent developments in myocardial protection, reperfusion damage, which ranges from myocardial stunning to cell death, still occurs and represents a significant cause of morbi-mortality (Stenestrand and Wal lentin, 2002).

Ischemia-reperfusion leads to myocardial injury through a variety of mechanisms that involve, for most part, mitochondrial dysfunction (Borutaite et al., 1996). It is well established that the mitochondrial respiratory chain represents a major subcellular source of reactive oxygen species (ROS) production during reperfusion of the ischemic myocardium (Lefer and Granger, 2000), and these species were expected to cause damage at or near the site of their formation. This burst of ROS production upon reperfusion leads to inhibition of oxidative phosphorylation (Nulton-Persson and Szweda, 2001) and increases the permeability of the inner mitochondrial membrane through the opening of the mitochondrial transition pore (MTP), a multiprotein complex formed at the contact site between the inner and outer mitochondrial membranes (Zoratti and Szabo, 1995). Such events are responsible for uncoupling of oxidative phosphorylation, δ-psi collapse, mitochondrial swelling, leading to the release of cytochrome c, activation of caspase pathways, and, ultimately, cell death by necrosis and/or apoptosis (Petronilli et al., 2001).

In this context, pharmacological interventions aiming at reducing mitochondrial dysfunction during cardiac ischemia-
reperfusion by regulating calcium homeostasis, or targeting mitochondrial ATP-dependent K⁺ channels, mitochondrial free radical production or the MTP may have therapeutic potential (for review, see Suleiman et al., 2001).

The peripheral benzodiazepine receptor (PBR), so called because it was initially discovered in peripheral tissues (Braestrup et al., 1977), is a 169 amino acid protein with five transmembrane domains associated with the mitochondrial outer membrane (Joseph-Liauzun et al., 1998). Photobueling studies indicate that this receptor is functionally associated with the voltage-dependent anion channel and the adenine nucleotide translocase (McEnery et al., 1992) and might be implicated in the regulation of MTP opening. PBR differs functionally from the central type benzodiazepine receptor because it lacks the coupling to γ-aminobutyric acid receptors and shows nanomolar affinity toward the benzodiazepine derivative Ro5-4864 (Syapin and Skolnick, 1979) and the isoquinoline carboxamide PK11195 (Le Fur et al., 1983), two compounds devoid of activity on the central benzodiazepine receptor. PBR tissue distribution analysis revealed an ubiquitous expression of this protein with a particularly high abundance in steroid-producing tissues but also in heart, liver, kidney, and blood cells (Anholt et al., 1986; Carayon et al., 1996).

PBR has been implicated in several mitochondrial functions and especially in the regulation of cholesterol transport into the mitochondria of steroid-producing tissues (Bernasau et al., 1993; Papadopoulos et al., 1997). In other tissues, its physiological role remains speculative. One putative function under cellular stress conditions has been suggested by Carayon et al. (1996) who demonstrated that the level of PBR expression was correlated with the resistance of the cell to oxidative stress and thereby protected mitochondria from radical damage. More recently, we demonstrated that PBR ligands reduced apoptosis induced by tumor necrosis factor-α in U937 cells (Bono et al., 1999).

However, as yet, there is no evidence as to whether PBR could be implicated in the protection of mitochondrial function and prevention of cardiac cell death after ischemia-reperfusion. We have investigated these questions by studying the effects of the potent, new, selective PBR ligand SSR180575 (Ferraz et al., 2002) in mitochondrial and cellular models of oxidative stress and in isolated tissue or in vivo cardiac ischemia-reperfusion models.

**Materials and Methods**

**Preparation of Rat Heart Mitochondria.** Male Sprague-Dawley rats (400–600 g; Janvier, Le Genest St. Isle, France) were anesthetized by an intraperitoneal injection of sodium pentobarbital (60 mg/kg) (Sanofi-Synthelabo, Gentilly, France). Hearts were removed, homogenized in ice-cold buffer [100 mM sucrose, 46 mM KCl, 10 mM TRIS, 2 mM EGTA, 0.5% (v/v) bovine serum albumin (BSA), 5 mM MgCl₂, 1 mM ATP, pH 7.2] containing 20% (v/v) protease (subtilisin Carlsberg; Sigma-Aldrich, St. Louis, MO) and incubated on ice for 5 min. To remove protease from the medium, the supernatant was then sedimented at 8,500 g during 15 min. The pellet was resuspended in the ice-cold buffer without protease. The homogenate was centrifugated at 500 g for 10 min. The supernatant was filtered through muslin and the mitochondria were sedimented at 8,000g for 15 min and then kept on ice in described buffer. Mitochondrial protein concentration was determined by the bicinchoninic acid method.

**Simultaneous Monitoring of Oxidative Phosphorylation Parameters.** Mitochondrial phosphorylation rate and membrane potential were measured at 25°C in a 3-ml glass vessel, respectively, with a high-sensitivity pH electrode and the lipophilic dye (mitophosphonium ion)-sensitive electrode. The incubation buffer contained 100 mM KCl, 40 mM sucrose, 10 mM KH₂PO₄, 5 mM MgCl₂, 1 mM EGTA, and 0.1% BSA (w/v). Mitochondrial protein concentration in the vessel was 350 μg/ml. Mitochondrial membrane potential and phosphorylation rate were calculated as described previously (Leduq et al., 1998).

**[³H]PBR Ligand Binding on Cardiomyoblasts and Heart Mitochondria.** Mitochondria were extracted as described previously and were resuspended in PBS containing 0.1% BSA at a final concentration of 50 μg/ml. H9C2 cardiomyoblasts from T75 flask were centrifuged at 1,500g for 5 min and washed once with PBS. After centrifugation, the cell pellets were resuspended in PBS containing 1% BSA at the concentration of 10³ cells/ml. [³H]Ro5-4864 or [³H]PK11195 (85 Ci/mmol) binding studies were performed on cell or mitochondria suspensions in 500 μl of PBS + 0.1% BSA at 4°C. Nonspecific binding was determined in the presence of 10 μM unlabeled corresponding ligand. After 20 min (mitochondria) or 1 h (cells), the assays were stopped by filtration through GF/C filters (Whatman, Maidstone, UK) and washed with 20 ml of binding buffer. Radioactivity trapped on the filters was determined by liquid scintillation counting. In both studies, total binding accounted for <10% of the radioligand added, whereas specific binding was >90% of the total binding at all concentrations of radiolabeled ligand used.

For competition experiments, increasing concentrations of unlabeled ligand (SSR180575, Ro5-4864, or PK11195) were added to a single concentration of [³H]Ro5-4864 or [³H]PK11195 (5 nM for cell suspension and 0.5 nM for mitochondrial suspension). In mitochondria, equilibrium was reached after 5 min (data not shown).

**Construction of PBR-Tagged Protein and Cell Transfection.** The receptor coding sequence from hPB11011 (Riond et al., 1991) with a 3′ sequence encoding the HA epitope (PYDVPDYA) was placed into the p658 vector (Miloux and Lupker, 1994) to give the pBM1784.

Culture dishes (35 mm) of subconfluent Cos7 or HEK293T (wild-type) cells were transfected with 1 μg of plasmid PBR-HA (BM1784) using FuGene (3 μl) (Roche Diagnostics, Mannheim, Germany). After 48 h, the transfected cells were replated in corresponding medium supplemented with geneticin (1 mg/ml). Colonies derived from single cells were picked and expanded as stably transfected cell lines. Transfected cell lines (Cos7-PBR-HA) were selected with geneticin (1 mg/ml).

**Cell Culture.** H9C2 and Cos7 cells were routinely cultured in Dulbecco’s modified Eagle’s medium and HEK293 cells (wild-type and transfected) in MEM. Both mediums were supplemented with 10% FCS and 4 mM glutamine.

**Protein Expression Studies.** Expression of epitope-tagged PBR (PBR HA) in transfected cells was analyzed by flow cytometry using anti-HA FITC, a mouse monoclonal IgG2a against tag HA (sc-7392; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**Laser Confocal Immunofluorescence Microscopy.** Cos7-PBR-HA cells (2 × 10⁵swell) were plated on collagen-coated culture slides for 24 h, fixed (45 min, 25°C), and permeabilized (45 min, 25°C) with Intrastain kit (DAKO, Bucks, UK). Cells were labeled with anti-HA FITC (Santa Cruz Biotechnology, Inc.), anti-cytochrome c monoclonal antibody FITC (Promega, Madison, WI), or anti-active caspase 3 FITC (BD Biosciences, San Jose, CA) antibodies. For qualitative determination of mitochondrial membrane potential, MitoTracker Red (Molecular Probes, Eugene, OR) was added to the cultures, and cells were processed for single and double labeling.

Confocal imaging was carried out using a PCM 2000 imaging system equipped with a Kr/Ar laser source (488- and 543-nm excitation) fitted to an Eclipse E-1000 microscope (Nikon, Tokyo, Japan). Images were collected with 60× PlanApo oil objective (Nikon) and...
treated by EZ2000 software. The pixel size was close to 0.45 μm and the axial resolution was 1 μm. FITC was excited at 488 nm and MitoTracker Red at 543 nm.

**Measurement of Apoptosis.** HEK293 cells were plated in 35-mm dishes (80 × 10^4 cells/well) in MEM culture medium + 10% FCS. After 24 h, cells were transfected with plasmid PBR-PA (BM1784). After 24 h, medium was removed and replaced by MEM without FCS and supplemented or not with H_2O_2 (30 μM) with or without SSR180575 (100 nM). After 24 h, apoptosis was measured with a photometric enzyme immunoassay (cell death detection enzyme-linked immunosorbent assay; Roche Diagnostics) for the quantitative detection of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes).

**Isolated Perfused Rat Hearts Submitted to Low-Flow Ischemia-Reperfusion.** Male Sprague-Dawley rats (300–350 g body weight) (Iffa Credo, L’Arbresle, France) were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and heparin (500 IU i.v.) administered. Under deep anesthesia, the hearts were excised, placed in ice-cold buffer, and mounted on a Langendorff perfusion system. Hearts were perfused retrogradely at constant flow (12 ml/min) with a Krebs-Henseleit bicarbonate buffer and gassed with 95% O_2, 5% CO_2, pH 7.4. Heart temperature was maintained at 37°C throughout the experiment. A latex isovolumic balloon was introduced in the left ventricle and inflated to give a preload of 5 to 10 mm Hg. Hearts were paced at 6 Hz except during the period of ischemia. Left ventricular developed pressure (LVEDP), perfusion pressure (PP), and heart rate (HR) were recorded throughout the experiment. After 20 min of stabilization, hearts were subjected to a 45-min normothermic low-flow ischemia (flow rate 1.2 ml/min) and reperfused at 12 ml/min for 30 min. Two experimental protocols have been used to test the effects of the PBR ligands SSR180575 and Ro5-4864 on the cardiac stunning phenomenon.

In the first (ex vivo) experiment, SSR180575 (0.3–30 mg/kg) or vehicle was administered orally 2 h before heart excision. In the second (in vitro) experiment, SSR180575 (0.01, 0.1, and 1 μM), Ro5-4864 (0.1 and 1 μM) or vehicle was added to the perfusate 5 min before and during reperfusion.

**Isolated Perfused Rabbit Heart Submitted to Regional Ischemia and Reperfusion.** Male New Zealand White rabbits (Iffa Credo) weighing between 2 and 2.5 kg were anesthetized with ketamine (120 mg/kg i.m.) and xylazine (10 mg/kg i.m.) and received heparin via a marginal ear vein (1,000 IU/kg). Through a midsternotomy, a 6-0 silk thread was passed through a 2-0 silk suture on a curved tapered needle. Both ends of the thread were passed through a polyethylene tube to form a snare. Myocardial infarction was induced by occluding the coronary artery for 30 min followed by 2 h of reperfusion. The body temperature was measured using a homeothermic blanket control unit and maintained at 37.5 ± 0.5°C during the experiment. At the end of the reperfusion period, rats were sacrificed by intravenously injection of a KCl-saturated solution. Hearts were excised and mounted on a Langendorff apparatus via the aorta. After 5-min washout with a saline solution, the left coronary artery was reoccluded and 1 ml of Evans blue dye was injected to define the area at risk. Hearts were then frozen in isopentane (−20°C) until infarct size measurements. SSR180575 (3, 10, and 30 mg/kg p.o.) or vehicle was administered 2 h before ischemia.

**Infarct Size Determination.** For infarct size measurement, hearts were sliced from apex to base into seven to eight transverse sections of approximately 1 mm for rats and 5 mm for rabbits. Sections were incubated at 37°C in 1% buffered 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) for 15 min, which allowed definition of the necrotic myocardium. Slices were placed in 10% formalin to enhance the contrast between stained and unstained tissue for 4 days and weighed. The infarct area (triphenyltetrazolium chloride negative) and the nonischemic area (Evans blue-stained area) were determined by computerized planimetry (Biscom). Infarct and area at risk weights were then calculated by multiplying each area by the slice weight and summing the products. The infarct size was expressed as the percentage of the area at risk.

**Statistical Analysis.** Data are expressed as mean ± S.E.M. Cardiac function data obtained on isolated hearts were assessed between experimental groups by a two-way ANOVA with repeated measure and Dunnett’s test was used for comparison. For infarct size and apoptosis data, a one-way ANOVA with a Dunnett test was used.

**Mitochondrial Studies**

**Oxidative Phosphorylation Parameters.** To mimic pathophysiological conditions, we studied mitochondrial function under oxidative stress induced by H_2O_2 (Table 1). Mitochondrial function was assessed by simultaneous measurements of mitochondrial membrane potential and phosphorylation rate under two distinct phosphorylating (state 3) or nonphosphorylating (state 4) states. H_2O_2 reduced in a dose-dependent manner the phosphorylation rate and the depolarization (data not shown). H_2O_2 was used at 1 mM to induce a rapid (within 2 min) and significant mitochondrial dysfunction. Under these conditions, H_2O_2 induced a large and significant decrease of phosphorylation rate (40–50%) and a significant fall in depolarization.

| Mitochondrial Studies | H_2O_2-induced mitochondrial dysfunction. IC_{50} values for SSR180575 and Ro5-4864 were 32 and 360 nM, respectively (Fig. 1), PK11195, a PBR antagonist, did not prevent H_2O_2-induced damage, but abolished the protective effect of SSR180575 (IC_{50} = 0.4 nM).

**Mitochondrial PBR Properties.** First, PBR protein was detected in mitochondrial preparation from rat heart by Western blot analysis (data not shown). Then, to rule out the possibility that H_2O_2 might directly modify PBR binding
propertie, $K_i$ values of PBR ligands were determined in presence or not of H$_2$O$_2$. In mitochondria isolated from the rat heart, $B_{\text{max}}$ and $K_P$ values were, respectively 2.5 ± 0.6 fmol/µg protein and 2.2 ± 0.8 nM when [³H]Ro5-4864 was used as a ligand. Under control conditions, the $K_i$ values of SSRI 0.575, Ro5-4864, and PK11195 were 1.5 ± 0.4, 4.2 ± 0.5, and 1.8 ± 0.4 nM, respectively ($n$ = 3). In the presence of H$_2$O$_2$, $K_i$ values remained unchanged at 2.5 ± 0.4, 2.3 ± 0.4, and 0.8 ± 0.1 nM, respectively ($n$ = 3). Similarly, the $B_{\text{max}}$ was not modified after H$_2$O$_2$ treatment (2.5 ± 0.6 versus 3.9 ± 2.5 fmol/µg protein after the treatment).

### Cellular Studies

To determine the effect of the PBR ligands on H$_2$O$_2$-induced cellular dysfunction, experiments were performed on cells transfected with the HA-tagged human PBR and on a rat cardiomyoblast cell line (H9C2) known to express PBR.

#### Cells Transfected with the Human-Tagged PBR-HA.

To validate our transfected cell model systems, we first characterized the expression and localization of the HA-tagged PBR. Then, we examined the cellular events induced by H$_2$O$_2$ by studying the key steps of the apoptotic process such as the mitochondrial events (membrane potential drop and cytochrome c release), caspase activation, and DNA fragmentation.

#### Characterization of PBR-HA Expression and Localization in Transfected Cells.

Cos7 “wild-type” cells were transfected with a plasmid containing the PBR-HA cDNA. Cells lines were stabilized from colonies as described under Materials and Methods. After fixation and permeabilization of the cells, the expression of PBR-HA was evaluated by flow cytometry using an anti-HA monoclonal antibody labeled with FITC (data not shown). The level cells expressing PBR-HA was close to 60%.

The intracellular localization of PBR-HA was determined by confocal microscopy experiments (Fig. 2I). Both mitochondria (red fluorescence with MitoTracker Red; Fig. 2Ia) and PBR-HA (green fluorescence; Fig. 2Ib) presented a perinuclear localization. Nontransfected cells did not present any green fluorescence (data not shown), demonstrating the specificity of the PBR-HA staining. When the images were merged (Fig. 2Ic), a colocalization between mitochondria and PBR-HA was clearly observed, confirming a mitochondrial localization of PBR-HA.

#### Measurement of the Events Linked to Apoptosis.

To determine whether PBR ligands could affect H$_2$O$_2$-induced apoptosis, mitochondrial membrane potential and cytochrome c distribution in Cos7 cells expressing or not PBR-HA

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**Table 1**

<table>
<thead>
<tr>
<th>Membrane Potential</th>
<th>Depolarisation</th>
<th>Phosphorylation Rate</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>State 3</td>
<td>State 4</td>
</tr>
<tr>
<td>Control (5)</td>
<td>104.3 ± 2.7</td>
<td>146.0 ± 1.6</td>
</tr>
<tr>
<td>H$_2$O$_2$ 1 mM (5)</td>
<td>101.4 ± 4.1</td>
<td>127.6 ± 6.3*</td>
</tr>
<tr>
<td>H$_2$O$_2$/SSR180575 1 µM (5)</td>
<td>101.0 ± 1.8</td>
<td>139.8 ± 2.3</td>
</tr>
<tr>
<td>H$_2$O$_2$/Ro5.4864 1 µM (5)</td>
<td>106.0 ± 4.1</td>
<td>142.5 ± 3.6</td>
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**Fig. 1.** Dose dependence of the protection by PBR agonists of mitochondrial dysfunction induced by H$_2$O$_2$. Rat heart isolated mitochondria, oxidizing complex I substrates (malate, glutamate + malonate) were pretreated with H$_2$O$_2$ and different concentrations of PBR ligands 2 min before addition of ADP. Phosphorylation rate was then measured and percentage values were calculated on corresponding control values (without H$_2$O$_2$ and PBR ligands). The results are given as the mean of 3 to 10 independent experiments ± S.E.M. Statistics analysis were done by ANOVA test (***, $p < 0.001$) and compared results obtained under H$_2$O$_2$ treatment in presence or absence of PBR ligands.
were analyzed by confocal microscopy after treatment with H₂O₂ (30 μM) with or without SSR180575 or Ro5-4864, 100 nM (Fig. 2II). After 16 h of H₂O₂ treatment, red fluorescence was absent revealing a collapse of Cos7 WT and Cos7-PBR-HA mitochondrial membrane potential (Fig. 2 IIb and IIC, respectively). Concurrently with the membrane potential fall, cytochrome c distribution occurred in the cytoplasm. A few cells did not present a total collapse of mitochondrial membrane potential, and as a consequence, kept a faint mitochondrial staining. Under these conditions, it seemed that cytochrome c and mitochondria were clearly not colocalized.

When Cos7-PBR-HA cells were treated with both H₂O₂ and a PBR ligand (SSR180575 or Ro5-4864, 100 nM; Fig. 2IIe), mitochondrial labeling was equivalent to that observed in the control conditions (Figure IIa), suggesting a normal membrane potential. In parallel, the PBR ligands prevented cytochrome c redistribution induced by H₂O₂. Thus, both cytochrome c release and mitochondrial membrane potential fall induced by a 16-h treatment with H₂O₂ were prevented by PBR ligands (SSR180575 or Ro5-4864; Fig. 2, IIe and IIB, respectively). In contrast, SSR180575 treatment of nontransfected Cos7 WT did not prevent the release of cytochrome c and mitochondrial membrane potential induced by H₂O₂ (compare Fig. 2, IIA and IIB), revealing an activation of caspase 3 that was markedly blunted by SSR180575 (100 nM; Fig. 2IIe). DNA fragmentation was evaluated by measuring oligonucleosomes production in a human cell line, HEK293, transfected with PBR-HA (40% expression as assessed by fluorescence-activated cell sorting analysis). H₂O₂ treatment (30 μM) significantly enhanced oligonucleosomes production (187 ± 16% of control), which was strongly reduced (51 ± 1% inhibition) in the presence of 100 nM SSR180575. In contrast, SSR180575 did not prevent DNA fragmentation induced by H₂O₂ in nontransfected cells.

Effect on the Cardiomyoblast Cell Line H9C2. Studies on cells transfected with the human tagged PBR presented above revealed a major role of PBR in prevention of H₂O₂-induced cell apoptosis. To confirm these results, we repeated these experiments in a rat cardiomyoblast cell line naturally expressing PBR, the characteristics of which were studied by radioligand binding experiments.

Determination of the Binding Characteristics of PBR in Cardiomyoblasts. In this study, both [³H]PK11195 and [³H]Ro5-4864 were used as PBR ligands (see Materials and Methods). Scatchard analysis of the saturation experiments with PK11195 or Ro5-4864 revealed a high-affinity and saturable site with both ligands: Kᵥ values were 12.2 ± 0.9 and 21.4 ± 1.6 nM, respectively, and B_max values were 2.38 ± 0.13 and 2.76 ± 0.16 × 10⁶ receptors/cell, respectively. Under the same conditions, competition experiments determined the specificity of the PBR receptor in cardiomyoblasts. Although Ro5-4864 and SSR180575, specific PBR ligands, rapidly displaced the binding of [³H]Ro5-4864 to PBR (IC₅₀ values (n = 3) were 15.5 ± 2.6 and 12.4 ± 1.0 nM, respectively), the effects of diazepam (ligand for both peripheral and central benzodiazepine receptors) and clonazepam (specific for the central benzodiazepine receptor) were very weak (IC₅₀ values were 374 ± 16 and >1000 nM, respectively). These results confirmed the “peripheral” specificity of benzodiazepine receptors identified in H9C2 cells.

Apoptosis in H9C2 Cells. In H9C2 cardiomyoblasts, H₂O₂ (30 μM) induced apoptosis, as revealed by monitoring oligonucleosomes production after 24 h. Under these conditions, PBR ligands prevented apoptosis induced by H₂O₂ in a dose-dependent manner. IC₅₀ values for SSR180575 and Ro5-4864 were 0.34 ± 0.05 and 0.47 ± 0.07 nM, respectively (n = 3), whereas nonspecific ligands presented a lower (diazepam, IC₅₀ = 47 ± 2 nM) or inexistent (clonazepam, IC₅₀ > 1000 nM) antiapoptotic effect. Blockade of the antiapoptotic
effect of SSR180575 by PK11195, a PBR antagonist (IC<sub>50</sub> values of SSR180575 in the absence or presence of PK11195 were 0.34 ± 0.05 and 762 ± 25 nM, respectively) confirmed that this effect was mediated by PBR.

Ischemia-Reperfusion Studies

Myocardial Stunning in Isolated Rat Hearts. In the ex vivo series of experiments, prior oral administration of SSR180575 led to a total immediate functional recovery (from 3 mg/kg), which was maintained during the 30-min reperfusion period. Heart rate-developed pressure product recovery expressed as percentages of preischemic values were as follows: control, 62 ± 12%; SSR180575 (3 mg/kg), 117 ± 3%; SSR180575 (10 mg/kg), 111 ± 5%; and SSR180575 (30 mg/kg), 112 ± 16% (p < 0.05 for all doses versus control) (Fig. 3). At the doses of 0.3 and 1 mg/kg p.o., SSR180575 did not show any significant protective effect (data not shown). SSR180575 pretreatment did not change basal hemodynamic parameters (data not shown).

To further evaluate the potential of a PBR ligand to reduce myocardial stunning when applied only during the reperfusion period, in vitro studies were conducted. SSR180575 (0.01, 0.1, and 1 μM) was added to the buffer just before reperfusion. Except for the lowest concentration, SSR180575 showed strong protective effects, leading to a total functional recovery comparable with that observed in ex vivo studies (Fig. 3). In the same experimental conditions, Ro5-4864 showed equivalent benefits from 1 μM (data not shown).

Myocardial Infarction in Isolated Rabbit Hearts. Perfusion of SSR180575 did not result in hemodynamic changes because baseline heart rate, coronary flow, and left ventricular developed pressure values for each of the groups were similar before regional ischemia (Table 2). Coronary flow was significantly reduced after occlusion of the coronary artery by approximately 50% in both groups, confirming that a similar degree of ischemia had been achieved in each group. Moreover, the rate-pressure product was reduced similarly in both groups during the occlusion period, suggesting a similar index of oxygen consumption. The recovery of the rate-pressure product was significantly improved by SSR180575 (1 μM) (control, 32 ± 12% versus SSR180575, 62 ± 4; p < 0.05). Moreover, SSR180575 significantly decreased both ischemic

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Fig. 3. Protective effect of SSR180575 administered p.o. at the indicated doses 2 h before heart excision (A) or added to the buffer just before reperfusion (B) on ischemia-reperfusion-induced left ventricular dysfunction (stunning) during the reperfusion phase in isolated rat hearts.
and reperfusion-induced rises in end-diastolic pressure (p < 0.05) (Fig. 4).

AAR expressed as a percentage of left ventricle area was approximately 50% for both groups. In this context, SSR180575 led to a marked infarct size reduction expressed as percentage of AAR from 42 ± 5% in the control group to 22 ± 3% in the treated group, p < 0.01 (Fig. 4).

Myocardial Infarction in the Anesthetized Rat. Figure 5 shows the effect of SSR180575 (3, 10, and 30 mg/kg p.o.) on infarct size after coronary occlusion/reperfusion. The ratio of area at risk to the left ventricle (AAR/LV) did not differ significantly among the groups (49 ± 2% in the control group, 48 ± 2% in the 3-mg/kg group, 50 ± 4% in the 10-mg/kg group, and 54 ± 3% in the 30-mg/kg group; not significant). SSR180575 decreased infarct size significantly at the two highest doses tested (control, 56 ± 5% versus SSR180575 (3 mg/kg), 43 ± 7%; not significant; SSR180575 (10 mg/kg), 31 ± 3%, p < 0.01; and SSR180575 (30 mg/kg), 37 ± 5%, p < 0.05).

**Discussion**

Mitochondria play a central role in cell function/survival by both regulating the ATP production necessary for normal cell processes and the induction of cell death via release of different apoptotic factors (Di Lisa and Bernardi, 1998). In the heart, irreversible alteration of both oxidative phosphorylation parameters (Borutaite et al., 1996) and mitochondrial membrane permeability (Griffiths and Halestrap, 1995) have been described as early events of the ischemia-reperfusion process. Mitochondrial membrane proteins are of particular importance in the conservation of mitochondrial function integrity. The PBR is located in the outer mitochondrial membrane and has been proposed to be a component of key membrane complexes such as the MTP. Consequently, it seemed interesting to determine the role of PBR and its ligands in mitochondrial, cellular and cardiac injury during ischemia-reperfusion.

To investigate the role of PBR and its ligands at the subcellular level, we developed models of oxidative stress (H2O2 treatment) in mitochondrial and cellular preparations. Oxidative stress is known to cause marked mitochondrial dys-function (Tsutsui, 2001). We have demonstrated, in a functional mitochondrial model, H2O2-induced inhibition of phosphorylation subsystem. Our observations on a functional model were well correlated with activity inhibition by H2O2 of the isolated ATPase enzyme pointed out by others (Lippe et al., 1993).

Although the damaging effects of H2O2 on mitochondrial function have been described, little is known about a possible preventative strategies leading to a preservation of cellular

**Fig. 4.** Summary of the data obtained on the regional ischemia-reperfusion in isolated rabbit hearts-mediated increased in left ventricular end diastolic pressure (LVEDP) (A) and decreased of left ventricular developed pressure × heart rate (LVDP × HR) (B) in control hearts (circles). SSR180575 (1 μM (squares) showed beneficial effects on cardiac function, as indi-cated by a smaller increase in LVEDP during ischemia-reperfusion and a better cardiac func-tion recovery during reperfusion. The fall in cor-onary flow (C) during regional ischemia was sim-ilar in both groups, *p < 0.05 versus control. Effect of SSR180575 (1 μM) on infarct size and area at risk measured after regional ischemia-reperfusion in perfused rabbit hearts (D). AAR (%LV) (area at risk expressed as a percentage of the left ventricle). INF (%LV) (infarct size expressed as a percentage of area at risk). INF/INF/AAR.
and organic functions. PBR has already been implicated in the resistance of hematopoietic cells to H$_2$O$_2$ treatment (Carayon et al., 1996). In this study, we have clearly demonstrated the protective and dose-dependent effect of two PBR ligands against H$_2$O$_2$ treatment in isolated cardiac mitochondria. In these conditions, the specificity of PBR agonist protection versus H$_2$O$_2$ was confirmed by antagonism with PK11195. PK11195 is a widely studied PBR ligand that was classified as an antagonist (Le Fur et al., 1983) and has been shown to block the antiapoptotic effects of Ro5-4864 in U937 cells (Bono et al., 1999). Thus, SSR180575 and Ro5-4864 prevent the major part of H$_2$O$_2$-induced impairment of oxidative phosphorylation and should allow an improved mitochondrial response to the ATP demand of cardiac cells in pathophysiological conditions.

During such pathophysiological processes, the role of the mitochondria is not limited to energy production, but also includes regulation of cell death/survival. After cellular stress, mitochondria integrate death signals through Bcl-2 family members and coordinate caspase activation subsequent to the release of cytochrome c (Desagher and Martinou, 2000). In agreement with other studies (von Harsdorf et al., 1996), H$_2$O$_2$ treatment resulted in many of the events demonstrated by Bono et al. (1999). Thus, SSR180575 and Ro5-4864 prevented the major part of H$_2$O$_2$-induced impairment of oxidative phosphorylation and should allow an improved mitochondrial response to the ATP demand of cardiac cells in pathophysiological conditions.

ROS generation, the aberrant exposure of phosphatidyserine residues on the plasma membrane surface, and nuclear fragmentation in the presence of different apoptosis inducers. These results could be explained by the inhibition by PK11195 of a basal antiapoptotic effect of endogenous PBR ligands such as protoporphyrin IX or other porphyrin derivatives.

Mitochondrial dysfunction (Lesniewsky et al., 2001) and programmed cell death have already been described as major causes of cardiac injury during ischemia-reperfusion. Mitochondria, by producing H$_2$O$_2$, participate in the oxidant load leading to myocardial stunning or infarction (for review, see Lefer and Granger, 2000). Myocardial stunning is characterized by a slowing of ventricular function recovery during reperfusion after reversible ischemia. Because SSR180575 prevented mitochondrial dysfunction caused by oxidative stress, we tested the ability of SSR180575 to reduce cardiac dysfunction after mild ischemia-reperfusion-induced myocardial stunning in isolated rat hearts. SSR180575 administered either in a preventive or in a curative manner, restored dose dependently the left ventricular function that was impaired during reperfusion. Similar protection was obtained with Ro5-4864, a structurally unrelated PBR ligand at a 10-fold greater dose. The implication of mitochondria in myocardial stunning has been the subject of a number of recent reports. Notably, it has been suggested that energy transfer or signaling between mitochondria and the site of energy use was impaired in stunned myocardium due to alteration of the functional coupling between the adenine nucleotide translocase and mitochondrial creatine kinase (Zuurvian and van Beek, 1997).

In myocardial infarction models, we showed that SSR180575 was able to reduce the infarct size either in isolated rabbit hearts or in anesthetized rats submitted to left coronary artery occlusion followed by reperfusion. The infarct size reduction observed in isolated rabbit hearts with SSR180575 was independent of coronary flow, because the fall in this parameter during coronary occlusion was comparable in both groups. Moreover, similar reductions in heart rate × left ventricular pressure product during coronary occlusion were measured in vehicle or SSR180575-treated hearts, demonstrating that the infarct size reduction was not due to a decrease in oxygen consumption during ischemia. A contribution of platelets and neutrophils could equally be ruled out because these experiments were performed on isolated fluid-perfused hearts. In the ex vivo isolated rat heart experiments reported in this article, oral administration of SSR180575 did not change the preischemic values of left ventricular pressure, LVDP, and perfusion pressure and we have demonstrated previously (our unpublished observations) that SSR180575 has no significant hemodynamic effects in several animal models. In addition, SSR180575 (0.001–10 μM) did not modify contractility or cardiac action potential parameters in the guinea pig isolated papillary muscle preparation (data not shown). Thus, we have not identified direct effects of SSR180575 on cardiac function or electrophysiology that could explain its cardioprotective properties.

In addition to the reduction of infarct size, SSR180575 also led to a 50% improvement in cardiac function recovery and partially prevented ischemic and reperfusion-induced contracture in isolated rabbit hearts. This rise in end-diastolic

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**Fig. 5.** Dose effect of SSR180575 administered p.o. 2 h before left coronary artery ligation/reperfusion in anesthetized rats on infarct size. *, $p < 0.05$ versus control; **, $p < 0.01$ versus control.
pressure, particularly during the ischemic phase, has largely been attributed to the combination of calcium overload and depletion of energetic pools (Steenbergen et al., 1990). Mitochondria are largely implicated in cellular calcium homeostasis especially during pathological events such as ischemia-reperfusion. For instance, opening of the mitochondrial K\textsubscript{ATP} channel (Wang and Ashraf, 1999; Murata et al., 2001) or inhibition of the MTP opening decreased mitochondrial calcium overload induced by ischemia-reperfusion in cardiomyocytes (Halestrap et al., 1997). Although, a precise role for PBR in mitochondrial calcium homeostasis has never been demonstrated, Salvetti et al. (2000) showed a rise of PBR levels inevitably caused an increase in the calcium concentration necessary to induce MPT opening on heart isolated mitochondria.

In parallel to disruption of mitochondrial calcium homeostasis, complexes of the mitochondrial oxidative phosphorylation apparatus seem to be damaged early in ischemia, and their function is further impaired during reperfusion (Veitch et al., 1992; Borutaite et al., 1993). The fact that SSR180575 preserved oxidative phosphorylation in mitochondria exposed to hydrogen peroxide could also, at least in part, explain the beneficial functional effects observed with this compound in hearts subjected to ischemia-reperfusion injury.

Necrosis and apoptosis seem to coexist in the ischemic heart (Anversa et al., 1997). Thus, infarct size reduction observed with SSR180575 could result from the reduction of either necrosis or apoptosis because triphenyltetrazolium chloride staining does not allow distinguishing these two forms of cell death. Because of its mitochondrial location, it is possible that PBR could be implicated in the control of apoptotic as well as necrotic cell death depending on the nature of stimuli. Similar results have been advanced regarding the role of MTP in the different cell death modes (Suleiman et al., 2001).

In conclusion, we have shown that a specific PBR ligand, SSR180575, protects the heart against mild and severe ischemia-reperfusion injury. These beneficial functional effects are coherent with our observation of a PBR-mediated action of this compound resulting in prevention of H\textsubscript{2}O\textsubscript{2}-induced impairment of cardiac mitochondrial oxidative phosphorylation and cardiomyoblast apoptosis.

These findings have significant conceptual and therapeutic implications. PBR ligands preserve mitochondrial integrity, minimize functional loss, and decrease apoptosis under oxidative stress conditions and protect the heart against ischemia-reperfusion injury. Therefore, potent PBR ligands such as SSR180575 may represent a novel therapeutic strategy against ROS-induced cardiac cell damage.

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