Involvement of Peripheral Benzodiazepine Receptor in the Oxidative Stress, Death-Signaling Pathways, and Renal Injury Induced by Ischemia-Reperfusion

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The peripheral benzodiazepine receptor (PBR) is a critical component of the mitochondrial permeability transition pore, which is involved in the regulation of cell death. In the present study we investigated the role of PBR in the regulation of signaling pathways leading to apoptotic and necrotic damage and renal dysfunction in a rat model of ischemia-reperfusion. Renal ischemia-reperfusion led to extended tubular apoptosis and necrosis that were associated with peroxidative damage, high levels of proapoptotic Bax expression, and low levels of antiapoptotic Bcl-2 expression, cleavage of death substrate, poly(ADP-ribose) polymerase (PARP), and activation of a key effector of apoptosis, caspase-3. Rat pretreatment with a novel PBR antagonist, SSR180575, significantly decreased postreperfusion oxidative stress and tubular apoptosis and necrosis. This effect was associated with inhibition of caspase-3 activation and PARP cleavage, upregulation of Bcl-2, and downregulation of Bax. Furthermore, inhibition of PBR accelerated the recovery of normal renal function, as assessed by measurement of levels of plasma creatinine and blood urea nitrogen. These findings reveal a role for PBR as a modulator of necrotic and apoptotic cell death induced by ischemia-reperfusion and suggest that regulation of PBR may provide new therapeutic implications for the prevention of acute renal failure.

The dysregulation of cell death and cell survival plays a key role in the pathogenesis of ischemia-reperfusion (I/R) injury. Cell damage induced by I/R triggers the activation of cascade of events leading to massive cell loss and organ dysfunction. The two forms of cell death involved in the postreperfusion tissue injury are apoptosis and necrosis (1). There is increasing evidence that mitochondria play a central role in executing different mode of cell death. Mitochondria are shown to produce reactive oxygen species (ROS) that can induce lipid peroxidation of cell membranes (2,3), protein and enzyme oxidation (4,5), and DNA damage (6,7), leading to apoptotic and necrotic cell death. It is well documented that mitochondria participate in the early steps of cell death through the opening of mitochondrial permeability transition pores (MPTP), the release of cytochrome c, apoptogenic factors, and changes in the balance of Bcl-2 family members (8,9). Mitochondrial dysfunction is associated with poly(ADP-ribose) polymerase (PARP) cleavage, an early sign of cell death after I/R (10). Between the two modes of cell death, apoptosis and necrosis, PARP activation seems to be more specifically linked to necrotic death after DNA damage because PARP$^{-/-}$ fibroblasts resist necrotic death induced by N-methyl-N'-nitro-N-nitrosoguanidine (11), and PARP$^{-/-}$ mice resist necrotic death during hemorrhagic shock (12). PARP inhibitors also prevent necrotic death induced by oxidants in thymocytes (13) or epithelial cells of intestine (14), kidney (15), and pulmonary artery (16), whereas they do not significantly affect oxidant-induced apoptosis. Therefore, the development of strategies to prevent mitochondrial damage and regulate cell death signaling pathways may lead to the definition of potential targets for therapeutic interventions.

The peripheral benzodiazepine receptor (PBR), a mitochondrial outer membrane protein, is functionally associated with the voltage-dependent anion channel and adenosine nucleotide translocase that form the mitochondrial permeability transition pore (MPTP). PBR tissue distribution analysis revealed an ubiquitous expression of the protein with a particularly high abundance in steroid-producing tissues (17). PBR is also abundant in lung, liver, kidney, and heart (18). Although this receptor has been characterized pharmacologically and biochemically, the functional assignment of PBR still has not been clearly defined. To date, the best documented role for PBR has been shown in steroidogenic tissues, where PBR mediates the

intramitochondrial cholesterol transport, which is the rate-limiting step in steroid biosynthesis (19). The disruption of the PBR gene in the R2C Leydig tumor cell line, a typical steroidogenic system, resulted in a dramatic decrease in the steroid biosynthesis (20). However, PBR is also expressed in cells that do not synthesize steroid; thus, the function of PBR could not be restricted to the steroidogenic function. Indeed, PBR has also been implicated in a number of cell functions including calcium channel activity, immune responses, porphyrin transport, and cell proliferation (21). Moreover, recent studies, including ours (22–24), showed that PBR is involved in the regulation of apoptosis. In the study presented here, we examined a role for PBR in tubular cell death and identified signaling pathways mediating postreperfusion tissue injury and renal dysfunction.

Materials and Methods

Materials

HBSS medium (Life Technologies-BRL, Eragny, France), polyvinylidene difluoride (PVDF) membrane (NEN Life Science Products, Boston, MA), rabbit polyclonal anti-PARP (Cell Signaling), rabbit polyclonal anti–Bcl-2, mouse monoclonal anti-Bax (Santa Cruz Biotechnology, Santa Cruz, CA), Kit ECL detector reagents (Amersham, Buckinghamshire, UK), Bio-Rad DC protein assay reagents (Bio-Rad Laboratories, Ivry-sur-Seine, France), REGM medium (Cambrex, East Rutherford, New Jersey), PBS (Life Technologies-BRL, Cergy-Pontoise, France), fetal calf serum (FCS), and glutamine (Boehrringer Mannheim, Meylan, Claix, France), ^{3}H-SSR180575 (specific activity: 86 Ci/mmol) (Amersham), SSR180575 (Sanofi-Synthelabo Recherche, Bagneux, France), SYTO-13, and propidium iodide (Molecular Probes, Eugene, Oregon). Other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Animals

All animal experiments were performed in accordance with the European Community Standards on the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Sanofi-Synthelabo Research. Male Sprague-Dawley rats weighing 200 to 250 g (Harlan ZI Du Malcourlet, France) were housed individually in standard laboratory cages with *ad libitum* access to food and water. The rats were anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally). Ischemia was induced by clamping of the right renal artery for 45 min with a nontraumatic vascular clip. Both kidneys were removed at 15 min or 6 h of reperfusion for further analysis. Sham-operated animals were subjected to the same surgical procedure without clamping the renal artery.

To measure renal function, both renal arteries were occluded for 45 min with a vascular clip. After surgery, the animals were returned to the cages, where they had free access to food and water. The rats were killed at 24 and 48 h of reperfusion, and plasma was collected from abdominal aorta at the time of death for measurement of creatinine and blood urea nitrogen (BUN). In addition to biochemical assessment of kidney function, removed kidney tissues were fixed in Dubosc solution and embedded in paraffin for histologic examination.

Experimental Protocols

In protocol 1, rats were subjected to unilateral ischemia by clamping the right renal artery for 45 min and were reperfused for 15 min or 6 h. In protocol 2, rats were subjected to bilateral ischemia by clamping the both renal arteries for 45 min and were reperfused for 24 and 48 h. For both protocols, sham operations were performed. Sham-operated animals and rats subjected to I/R were treated with vehicle or SSR180575 (10 mg/kg intraperitoneally) 30 min before ischemia. The dose of SSR180575 was based on our previous study in an animal model (25).

Determination of Malondialdehyde

Malondialdehyde (MDA), an end product of lipid peroxidation, was assessed according to Aruoma *et al.* (26) with some modifications. The kidneys obtained after 15 min of reperfusion were placed in 50 mM Tris-HCl (pH 7.4) containing 180 mM KCl and 10 mM EDTA in a total volume of 2 ml and homogenized. Briefly, 20 μl homogenate were added to 980 μl water, 100 μl HCl (0.5 M), and 1 ml 0.8% thiobarbituric acid. This solution was heated to 95°C for 20 min. After addition of 2 ml of butanol-1, the mixture was centrifuged at 2000 rpm for 10 min at 4°C. The absorbance of the upper layer was read at 548 nm (Spectrofluo JY3 D, Jobin Yvon, Paris, France). MDA bis-dimethyl acetal was used as the external standard. Results are expressed as nanomoles MDA per milligram protein.

Evaluation of Postischemic Apoptosis

DNA fragmentation was visualized *in situ* on Dubosc-fixed and paraffin-embedded sections by the TUNEL (*t*erminal transferase-mediated d*U*TP *n*ick-*e*nd *l*abeling) procedure with the apoptosis detection kit of Promega (Madison, WI). Briefly, deparaffinized sections were incubated in a 20 μg/ml proteinase K solution to permeabilize the tissues, then rinsed. The sections were incubated with 1 μl of terminal deoxynucleotidyl transferase (25 U/μl) and fluorescein-12-dUTP in equilibration buffer (25 mM Tris-HCl, pH 6.6; 200 mM potassium cacodylate, pH 6.6, 2.5 mM cobalt chloride, 0.25 mg/ml BSA, 0.2 mM DTT) for 1 h at 37°C. Then after rinsing in SSC and PBS, the slides were immersed in 40 ml propidium iodide solution (1 μg/μl) for 15 min. The positive control was treated by DNAse I (1 μg/μl) before being processed with the TUNEL procedure.

Detection of Caspase-3 Activity

The activity of caspase-3 was determined by use of a fluorescence substrate, Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC; Bachem), as described previously (27). Briefly, the frozen kidney cortices were homogenized with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES, pH 7.4), containing 0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 42 mM KCl, 5 mM MgCl$_2$, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, and 1 μg/ml pepstatin A. The homogenate was then centrifuged at 10,000 $\times$ g for 10 min. Supernatant (containing 250 μg total protein) was incubated with 40 μM of the caspase-3 fluorescence substrate Ac-DEVD-AMC for 60 min at 37°C. At the end of incubation, substrate cleavage was monitored fluorometrically with a spectrofluorometer (Spectrofluo JY3 D, Jobin Yvon, Paris, France) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Data are expressed as arbitrary units per milligram of protein. One unit of enzyme activity is defined as amount of enzyme required to cleave 40 μM of Ac-DEVD-AMC. Homogenate protein was measured by the Bradford method, as described in the Bio-Rad protein assay kit.

Western Blot Analysis

Frozen tissue samples were homogenized in tissue lysis buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM phenylmethylsulfonyl flu-

oride, 1 mM benzamidine, 10 μg/ml PMSF, 3 μg/ml aprotinin and 3 μg/ml leupeptin]. After sonication, 30 μg of proteins were loaded on a 10% (for PARP) or on a 12% (for Bax and Bcl-2) polyacrylamide gel and transferred to PVDF membrane. The membrane was blocked with 1% BSA in TBS-Tween 20 (0.1%) overnight at 4°C. Polyclonal anti-PARP (1:1000), monoclonal anti-Bax (1:100), or polyclonal anti–Bcl-2 (1:200) was used as primary antibody. After incubation with appropriate horseradish peroxidase–linked secondary antibody (1:10,000 for 30 min at room temperature), proteins were detected by ECL reaction. The blot was stripped completely of antibodies before reprobing with a polyclonal anti-actin (1:1000) antibody used as a standard.

Renal Function

Creatinine and BUN were measured with a Vitros 950 Autoanalyzer (Clinical Diagnostics).

Histologic Examination

Dubosc-fixed, paraffin-embedded kidney specimens were sectioned at 4 μm and stained with hematoxylin-eosin. Histologic changes were evaluated by measuring tissue necrosis graded on a 0 to 5 scale in relation to the extent of kidney damage: 0 = none; 1 = up to 10%; 2 = from 10% to 25%; 3 = from 25% to 50%; 4 = from 50% to 75%; 5 = more than 75%. Tubular necrosis was assessed at 24 and 48 h after I/R.

^{3}H-SSR180575 Binding on Renal Proximal Tubule Epithelial Cells

Human renal proximal tubule epithelial cells (RPTEC) were obtained from Clonetics (San Diego, CA) and cultured in REGM supplemented with 10% heat-inactivated FCS and 4 mM glutamine at 37°C (5% CO_2, 95% air atmosphere). Before binding experiments, cells were washed once in PBS, isolated by accutase, centrifuged at 400 × g for 10 min, and resuspended at the concentration of 7.10^4 cells/ml in binding buffer (PBS containing 0.1% BSA). ^{3}H-SSR180575 binding studies were performed on cell suspensions in 500 μl of PBS + 0.1% BSA at 4°C, respectively. Nonspecific binding was determined in the presence of 10 μM of unlabeled SSR180575. The assays were stopped after 1 h by filtration through Wathman GF/C filters and washed with 12 ml of binding buffer. Radioactivity trapped on the filters was quantified by liquid scintillation counting.

Evaluation of Cell Apoptosis and Necrosis

RPTEC were plated at a density of 15 × 10^3 cells/well in 24-well plates and incubated for 24 h in serum-free REGM. Cells were pretreated with or without SSR180575 (100 nM) and treated with H_2O_2 (50 μM) for 24 h. Necrosis and apoptosis were evaluated by fluorescence staining with vital fluorescent dyes: 0.6 μM SYTO-13 (a permeant DNA intercalating green-colored probe) and 15 μM propidium iodide (a nonpermeant intercalating orange probe) as described by Meilhac et al. (24). Cells were counted with an inverted fluorescence microscope (Fluovert FU, Leitz). Normal nuclei exhibited loose chromatin colored green by SYTO-13; apoptotic nuclei exhibited condensed green-colored chromatin (postapoptotic necrosis characterized by nuclei exhibiting the same apoptotic morphologic features, but with red color) chromatin; necrotic cells exhibited red-colored nuclei with loose chromatin.

Statistical Analyses

The data are presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA, followed by Newman-Keuls multiple comparisons. P values less than 0.05 were considered statistically significant.

Results

Postischemic Oxidative Stress

We and other investigators have previously demonstrated that the early phase of reperfusion is associated with enhanced oxidative damage (28,29). To determine the involvement of PBR in postreperfusion oxidative stress, we tested the effect of the PBR agonist SSR180575 on the renal levels of a marker of peroxidative damage, MDA, in rats subjected to unilateral ischemia or to sham surgery. After 15 min of reperfusion, renal content of MDA was increased in vehicle-treated ischemic kidneys of rats compared with those of sham-operated animals (Figure 1). Pretreatment with SSR180575 markedly prevented postischemic MDA increase (Figure 1).

Postischemic Renal Apoptosis

We next determined whether prevention of oxidative stress by the PBR agonist was associated with a modification of postreperfusion cell apoptosis. Apoptosis was evaluated by detection of fragmented chromosomal DNA by the TUNEL assay and the measure of activity of caspase-3 at 6 h of reperfusion. This time has been chosen on the basis of our previous studies showing that tubular apoptosis and apoptotic factors can precisely quantified after 6 h of reperfusion (27). Figure 2 shows representative sections of kidneys from sham-operated and vehicle- or SSR180575-treated ischemic rats. In sham-operated rats, TUNEL-positive cells were undetectable. In contrast, kidney sections from rats submitted to I/R showed
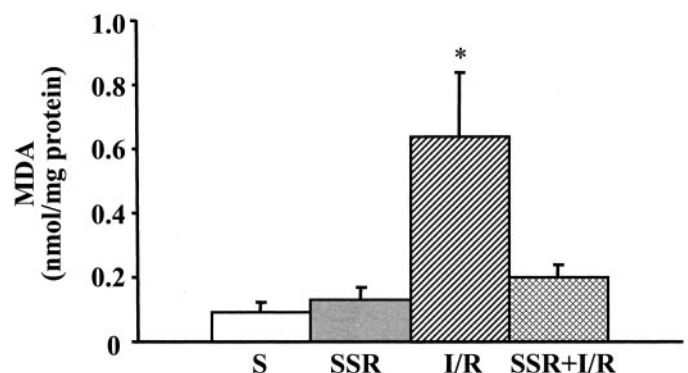

Figure 1. Renal postreperfusion lipid peroxidation after SSR180575 treatment. Malondialdehyde (MDA), a lipid peroxidation product, was measured by the thiobarbituric acid assay in renal cortex homogenate 15 min after reperfusion. Sham-operated and ischemic groups of rats were treated with vehicle or SSR180575 (10 mg/kg, intraperitoneally) 30 min before 45 min of unilateral ischemia. Data are expressed as the mean ± SEM from four separate experiments. $^*P < 0.01$ *versus* sham group. S, sham-operated rats treated with vehicle ($n = 4$); SSR, sham-operated rats treated with SSR180575 ($n = 4$); I/R, rats subjected to ischemia followed by reperfusion ($n = 5$); SSR+I/R, rats treated with SSR180575 before ischemia followed by reperfusion ($n = 5$).
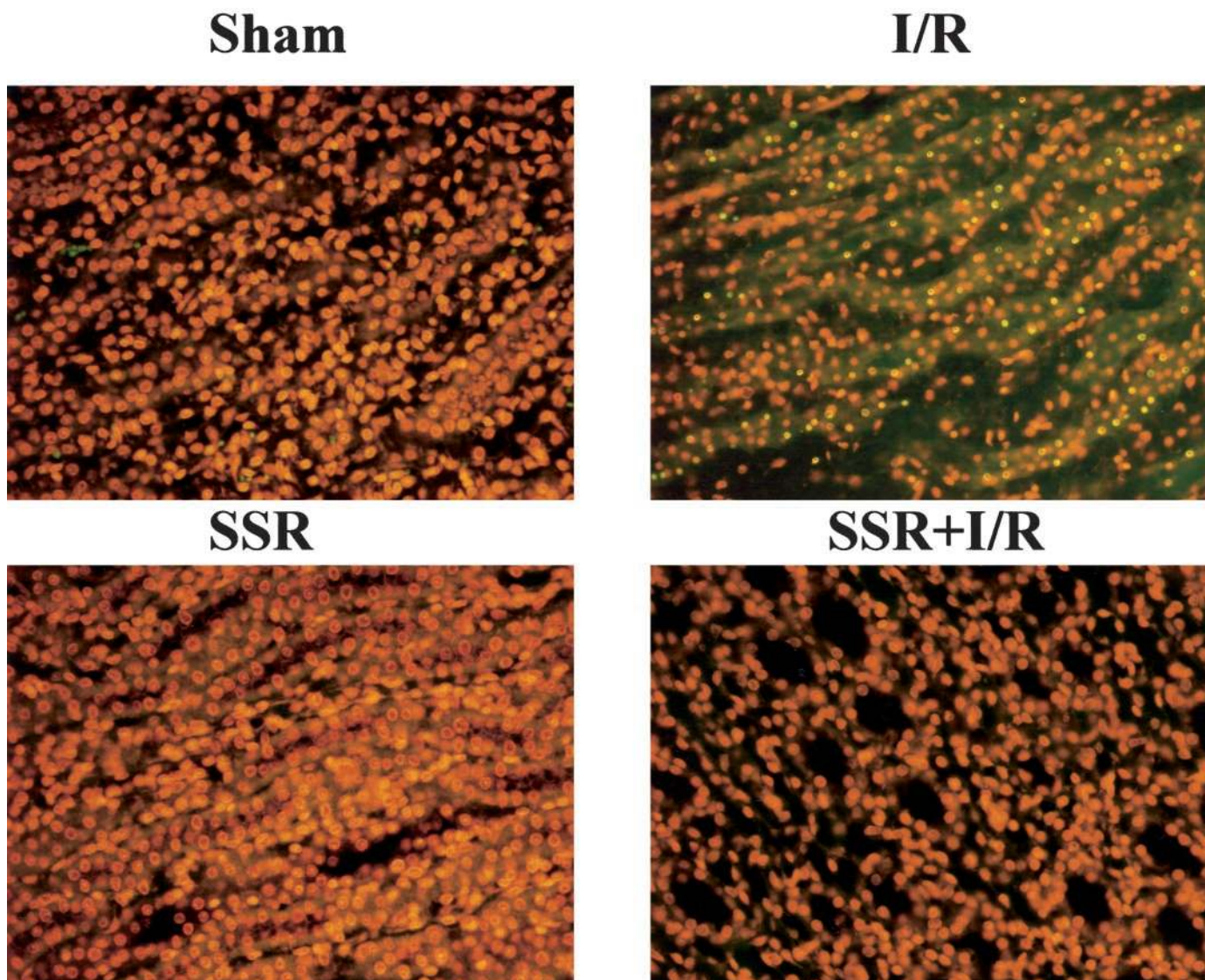
Figure 2. Effect of SSR180575 pretreatment on postreperfusion cell apoptosis. Apoptosis was evaluated by TUNEL staining of kidney sections after 6 h of reperfusion. Light photomicrographs (original magnification, ×40) of kidney sections from the sham-operated and ischemic groups of rats pretreated with vehicle or SSR180575. The bright green dots correspond to a representative TUNEL-positive (fluorescence) nucleus. $^*P < 0.001$ *versus* sham-operated group; $^\#P < 0.001$ *versus* ischemic group. S, sham-operated rats treated with vehicle ($n = 4$); SSR, sham-operated rats treated with SSR180575 ($n = 4$); I/R, rats subjected to ischemia followed by reperfusion ($n = 7$); SSR+I/R, rats treated with SSR180575 before ischemia followed by reperfusion ($n = 7$).

a significant tubular apoptosis (TUNEL-positive cells: $17\% \pm 2\%$ of total number of cells) (Figure 2). Rat treatment with SSR180575 before I/R completely prevented tubular apoptosis (Figure 2). The involvement of PBR in tubular apoptosis was confirmed by the measure of caspase-3 activity. Indeed, the increase in caspase-3 activity observed in vehicle-treated animals submitted to I/R was fully prevented by SSR180575 pretreatment (Figure 3).

To further characterize the role of PBR in the postreperfusion cell death pathway, we examined the effects of SSR180575 on expression of the proapoptotic Bax and the antiapoptotic Bcl-2 proteins by Western blot analysis. As shown in Figure 4, the immunoreactivity of Bax protein significantly increased in ischemic kidneys after 6 h of reperfu-

sion. The increase in Bax amount was concomitant to the decrease in the expression of the antiapoptotic protein Bcl-2. These changes in Bax and Bcl-2 expression were completely prevented by SSR180575 pretreatment.

Finally, to determine whether the regulation of tubular cell death by PBR may involve cleavage of PARP, an early marker of cell death, we measured PARP cleavage by Western blot analysis. PARP cleavage occurred 6 h after reperfusion in vehicle-treated animals. This effect was fully prevented by rat treatment with SSR180575 before I/R.

Histomorphological and Functional Studies

We next investigated the role of PBR in necrotic tubular cell death after I/R. Experiments were performed in kidneys from
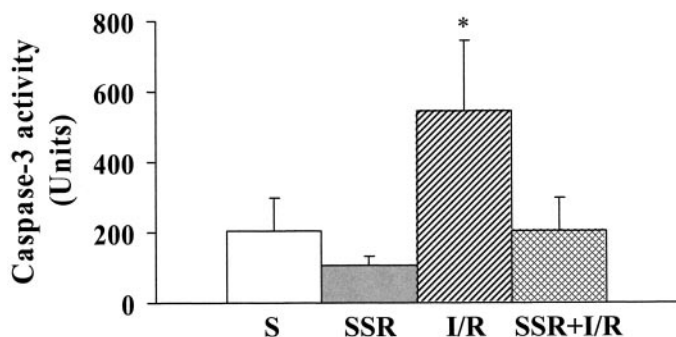
Figure 3. Caspase-3 activity in postreperfusion rat kidney after SSR180575 treatment. Caspase-3 activity was measured in supernatant by the fluorometric assay with substrate Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) in vehicle-treated and SSR180575-treated rats subjected to unilateral ischemia for 45 min or to sham operation. Rats were killed at 6 h after reperfusion. One unit of enzyme activity is defined as amount of enzyme required to liberate 40 μM of Ac-DEVD-AMC for 60 min at 37°C. $^{*}P < 0.01$ *versus* sham group. S, sham-operated rats treated with vehicle ($n = 4$); SSR, sham-operated rats treated with SSR180575 ($n = 4$); I/R, rats subjected to ischemia followed by reperfusion ($n = 5$); SSR+I/R, rats treated with SSR180575 before ischemia followed by reperfusion ($n = 6$).
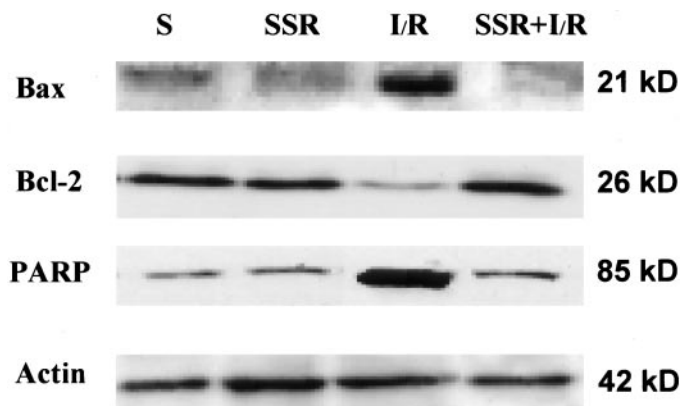

Figure 4. Expression of Bax, Bcl-2, and poly-(ADP-ribose)-polymerase (PARP) in postischemic kidneys. Kidneys from sham-operated rats treated with vehicle (S) or SSR180575 (SSR) and ischemic kidneys from rats treated with vehicle (I/R) or SSR180575 30 min before ischemia (SSR+I/R) were removed after 6 h of reperfusion. The protein expression of Bax, Bcl-2, and PARP was determined by Western blot in whole tissue lysates. Equal protein loading was displayed by actin. The blots are representative of the results obtained from four animals.

rats submitted to bilateral ischemia followed by 24 and 48 h of reperfusion. We have previously shown this model is appropriate to evaluate both tubular necrosis and renal dysfunction (27).

Histologic sections of kidneys from vehicle-treated ischemic rats showed extensive tubular necrosis mainly located at the proximal tubules (Figure 5 and Table 1). In contrast, the histology of ischemic kidneys from rats pretreated with SSR180575 was indistinguishable from sham-operated animals (Figure 5 and Table 1). Treatment with SSR180575 in sham-operated animals did not produce any detectable histologic abnormalities. Kidney function was evaluated by determining creatinine and BUN levels at 24 h and 48 h after reperfusion. Ischemia followed by reperfusion produced significant increases in plasma concentrations of creatinine and BUN (Figure 6). However, rat pretreatment with SSR180575 significantly decreased creatinine and BUN concentrations after 24 and 48 h of reperfusion. SSR180575 administration 2 h after reperfusion did not prevent tissue injury and renal dysfunction (data not shown). These results suggest that PBR play a critical role in the early phase of reperfusion.

Effect of SSR180575 on H_2O_2-Induced Cell Death in RPTEC

To determine the potential relevance of PBR in tubular damage in human, we verified the expression of PBR in RPTEC and we tested their involvement in H_2O_2-dependent cell death. Radioligand binding studies showed that the total binding of ^{3}H-SSR180575 to RPTEC was dose dependent. Furthermore, the nonspecific binding was linearly dependent on the concentration of ^{3}H-SSR180575 and did not exceed 10% of the total binding (Figure 7A). The Scatchard analysis of these data (Figure 7B) demonstrated the presence of a specific binding site of SSR180575 with a Bmax of $5.63 \pm 0.34 \cdot 10^6$ receptors per cell and a Kd of 3.05 ± 0.28 nM on RPTEC.

Apoptotic and necrotic cells were discriminated by green (SYTO-13) and orange (propidium iodide) staining, respectively. Cell treatment with H_2O_2 (50 μM) for 24 h induced a significant increase in the number of SYTO-13 and propidium iodide–stained cells as compared with untreated cells (Figure 8, A and B). Pretreatment with SSR180575 (100 nM) significantly decreased H_2O_2-induced cell apoptosis and necrosis (Figure 8B).

Discussion

The study presented here provides strong evidence that PBR, a mitochondrial outer membrane protein, plays an important role in the modulation of signaling pathways mediating tubular apoptotic and necrotic cell death and renal dysfunction after I/R. We also demonstrate that SSR180575, a novel specific PBR agonist (23), has a preventive effect against I/R injury in rat kidneys.

There is increasing evidence that apoptosis plays an important role in the pathogenesis of I/R injury. We and other investigators have demonstrated that inhibition of apoptosis during I/R is associated with reduction of postreperfusion damage and improved renal function and survival (30). It is well established that the mitochondrial dysfunction, which occurs during I/R, represents a key mechanism by which cells are damaged. Mitochondria are intimately involved in apoptosis because of its metabolism, the principal source of high energy intermediates, but also of its role in ROS production, which can have both direct and indirect effects on apoptosis. ROS are proposed to initiate early triggering events in the postreperfusion cell damage. In the attempt to define the role of
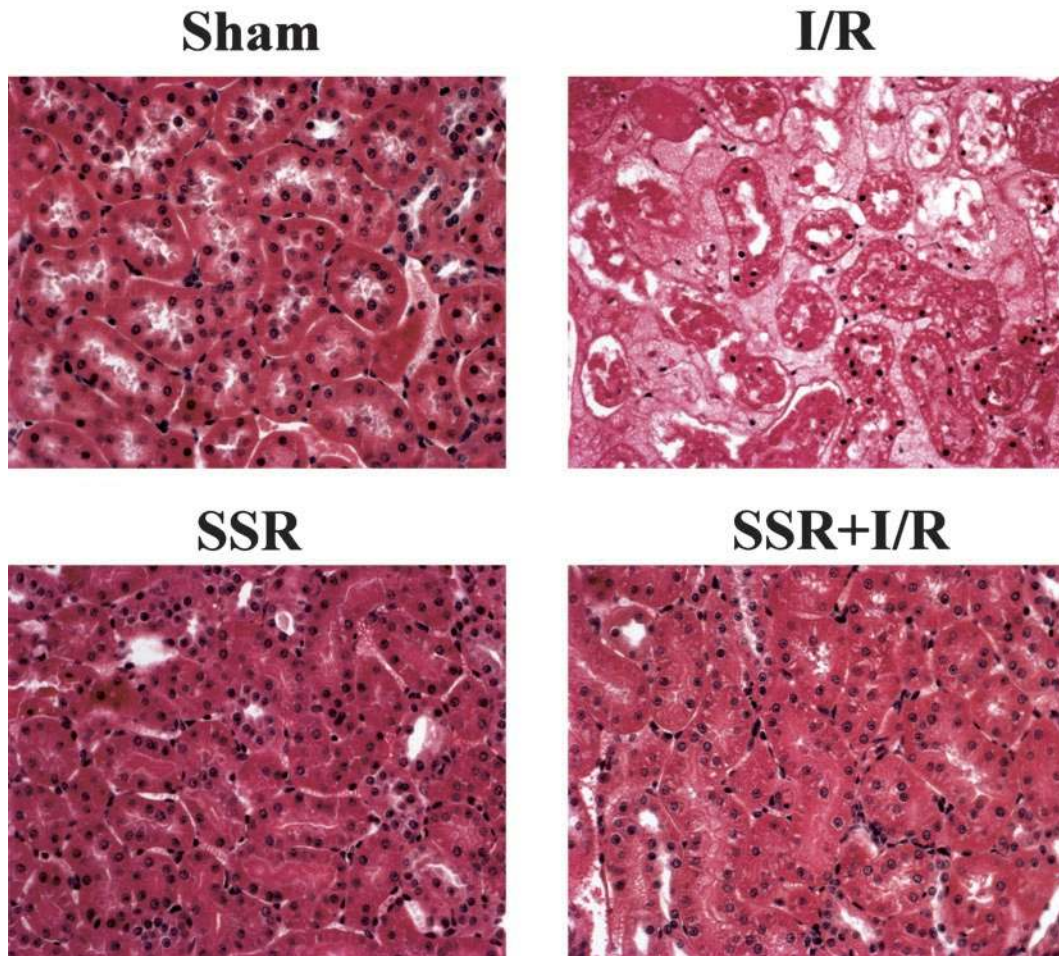
Figure 5. Histomorphology of ischemia-reperfused kidneys. Rats were killed after 24 and 48 h of renal reperfusion. Light photomicrographs (original magnification, ×40) of hematoxylin-eosin–stained sections of kidney from sham-operated rats treated with vehicle (S) or SSR180575 (SSR) and ischemic kidney from rats treated with vehicle (I/R) or SSR180575 before ischemia (SSR+I/R). Results are representative of five to eight independent experiments.

Table 1. Scoring of tubular necrosis induced by ischemio-reperfusion in rats treated with vehicle (I/R) or SSR180575 (SSR + I/R)

Tubular Necrosis	R = 24 h		R = 48 h	
	I/R	SSR + I/R	I/R	SSR + I/R
0%	—	3	—	1
0–10%	—	4	—	3
10–25%	1	1	—	—
25–50%	1	—	3	—
50–75%	3	—	2	—
>75%	1	—	—	—

PBR in the regulation of apoptosis, we have shown that treatment with a PBR agonist attenuates oxidative stress induced by I/R and prevents tubular cell apoptosis in *in vitro* and *in vivo* conditions. These findings are consistent with earlier observations showing the role of PBR in oxidative processes by the involvement of this receptor in the protection of hematopoietic cells against apoptosis after H_2O_2 treatment (31). In that study, the expression of PBR and the resistance of hematopoietic cells to H_2O_2 toxicity were found to be correlated and resistance of cell to H_2O_2 significantly increased by transfection with PBR. In the same way, we previously showed the protective effect of PBR agonists treatments on a model of H_2O_2-induced oxidative stress on HEK 293T transfected with a human form of PBR (24) or human lymphoblastoid cell line U937 (32). Thus, the PBR may be involved in at least two aspects of mitochondrial oxidative processes: the protection of mitochondria from exogenous ROS and the regulation of mitochondrial generation and/or release of ROS.

In addition to our previous demonstration of the role of PBR in cardiomyoblast apoptosis and cardiac cell damage (23), our study expands knowledge about the mechanisms involved in the apoptotic and necrotic cell death in the kidney. Indeed, we showed that PBR modulates the expression of the proapoptotic Bax and antiapoptotic Bcl-2 proteins. The relative proportion of pro- and antiapoptotic proteins appears to be an important contributing factor in determining cellular death and survival. Increased expression of the proapoptotic protein Bax has been
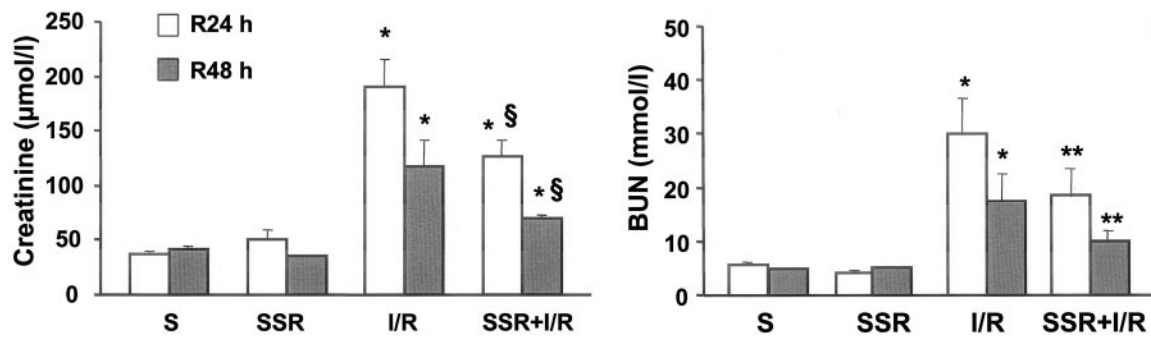
Figure 6. Effects of SSR180575 on renal function. Renal function was evaluated by determining plasma creatinine (**A**) and blood urea nitrogen (BUN) (**B**) in vehicle-treated and SSR180575-treated rats subjected to bilateral ischemia followed by 24 h and 48 h of reperfusion. Data shown are mean $\pm$ SEM from five independent experiments. $^*P < 0.01$ *versus* sham group; $^{**}P < 0.05$ *versus* I/R group; $^§P < 0.01$ *versus* I/R group. S, sham-operated rats treated with vehicle ($n = 4$); SSR, sham-operated rats treated with SSR180575 ($n = 4$); I/R, rats subjected to ischemia followed by reperfusion ($n = 5$); SSR+I/R, rats treated with SSR180575 before ischemia followed by reperfusion ($n = 5$).
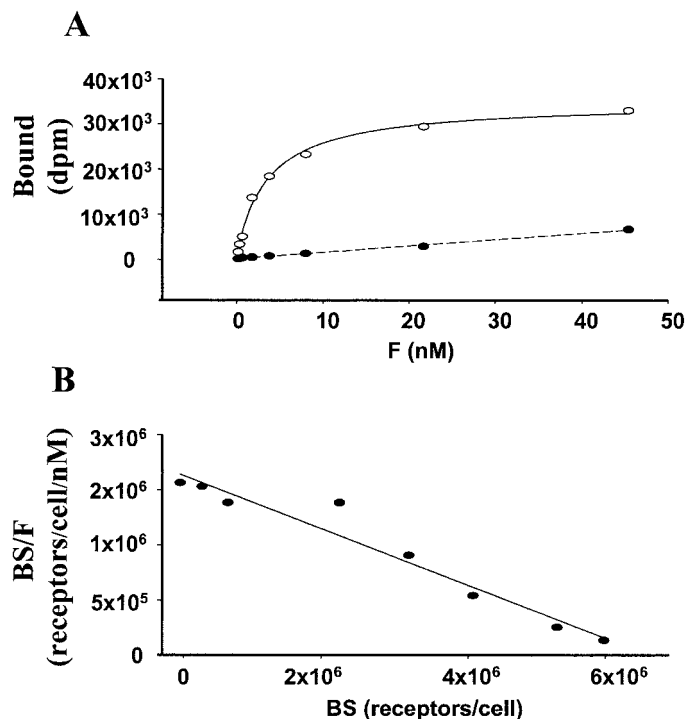

Figure 7. Binding characteristics of ^{3}H-SSR180575 in RPTEC. (A) Saturation curve: RPTEC (17.5×10^3 cells) were incubated for 60 min at 4°C with increasing concentrations of ^{3}H-SSR180575. Nonspecific binding is determined in the presence of 10 μM unlabeled SSR180575. (B) Scatchard plot of the specific binding of ^{3}H-SSR180575 was calculated from saturation isotherms.

found in experimental models of acute renal failure (33). In contrast, Bcl-2 expression is reported to be down-regulated after ischemic renal damage (34). Studies have revealed that overexpression of Bcl-2 prevents MPTP opening and inhibits cytochrome c release to impede the progression of the apoptotic pathway (34). Indeed, we have previously demonstrated the role of PBR in apoptosis through early mitochondrial events including decrease of the mitochondrial membrane potential, release of cytochrome c leading to caspase-3 activation and

DNA fragmentation (23). Taken together, these results show that PBR may regulate different mitochondria functions involved in cell death processes.

The study presented here provides the first *in vivo* evidence that PBR plays a crucial role in the PARP activation pathway. In particular, we have shown that treatment with a PBR agonist prevents I/R-induced PARP cleavage, an early sign of cell death. PARP is a nuclear nick sensor enzyme that becomes activated in response to DNA damage (35). Activated PARP cleaves NAD^+ into nicotinamide and ADP-ribose, and catalyzes the latter on nuclear acceptor proteins such as histones and PARP itself (35). Excessive activation of PARP depletes the cellular NAD^+ and ATP pools and causes necrotic cell death (36). The fact that rat pretreatment with SSR180575 completely prevents postreperfusion PARP cleavage suggests that PBR is involved in the cascades of PARP-mediated cell events. Further studies are needed to elucidate the precise mode of action of the PBR in necrotic pathways after I/R.

Functional studies show that, in addition to the protective effects against renal injury, PBR ligand also improves the recovery of renal function. Indeed, we have demonstrated that rat pretreatment with SSR180575 accelerated the restoration of normal creatinine and BUN levels. In addition, we have found that the improvement in renal function was concomitant to histologic evidence for the beneficial effect of PBR in tubular cell necrosis. These results are in agreement with previous reports showing that inhibition of cell death often leads to improved function in many organ systems (37–40). It is noteworthy that, in an autotransplantation pig kidney model, PBR expression correlates with the quality of kidney preservation and might serve as an index of kidney and mitochondria viability (41). These results, along with those presented here, suggest that prevention of mitochondrial damage by a PBR agonist confers a protective effect on overall tubular integrity and renal function after I/R.

In conclusion, we have demonstrated, by means of an *in vivo* model of renal I/R, the involvement of PBR in the control of PARP-mediating signaling pathways leading to tubular apoptosis and necrosis and renal dysfunction after I/R. These
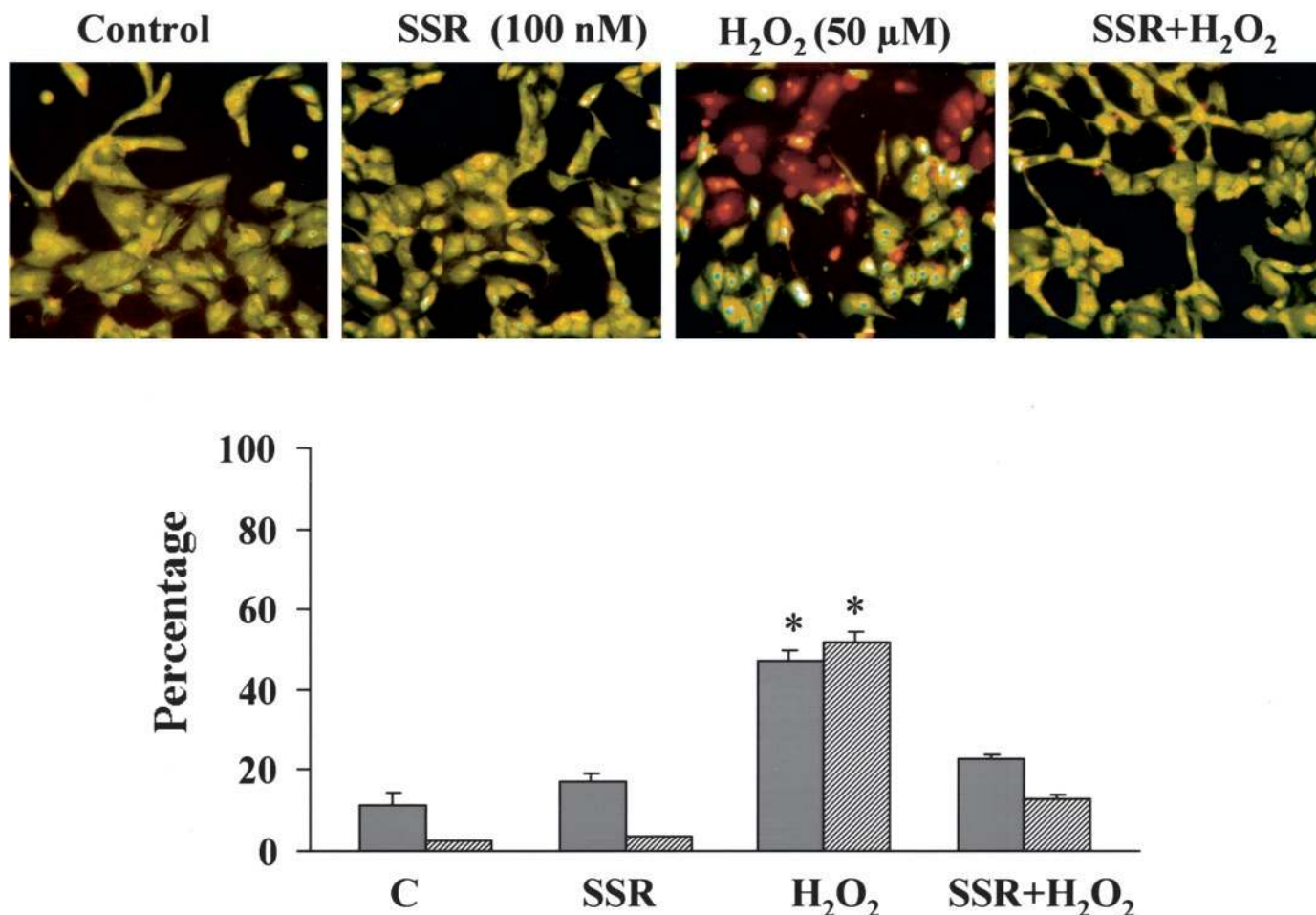
Figure 8. Morphologic analysis of H_2O_2-induced tubular cell apoptosis and/or necrosis in RPTEC. Cell nuclei were double stained with SYTO-13 and propidium iodide, discriminating normal cells from cells undergoing apoptosis or necrosis by fluorescence microscopy. (A) Photomicrographs of human renal proximal tubule epithelial cells pretreated for 20 min with or without SSR180575 before 50 μM H_2O_2 addition for 24 h. (B) Quantification of renal tubule epithelial cells stained by SYTO-13 and propidium iodide. The data result from counting of three fields of around 100 cells each per dish. Values are mean $\pm$ SEM of the number of morphologically apoptotic cells relative to the control from three separate experiments ($*P < 0.05$ *versus* control).

findings provide new insight into the mechanisms involved in the postreperfusion apoptotic and necrotic cell death by this receptor and may have therapeutic implications for the prevention of acute renal failure.

Acknowledgments

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