Adrenalectomy prevents renal ischemia-reperfusion injury

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Ramírez V, Trujillo J, Valdes R, Uribe N, Cruz C, Gamba G, Bobadilla NA. Adrenalectomy prevents renal ischemia-reperfusion injury. Am J Physiol Renal Physiol 297: F932–F942, 2009. First published August 5, 2009; doi:10.1152/ajprenal.00252.2009.—Spironolactone treatment prevents renal damage induced by ischemia-reperfusion (I/R), suggesting that renoprotection conferred by spironolactone is mediated by mineralocorticoid receptor (MR) blockade. It is possible, however, that this effect is due to other mechanisms. Therefore, this study evaluated whether adrenalectomy prevented renal damage induced by I/R. Three groups of Wistar rats were studied: 1) a group subjected to a sham surgery, 2) a group subjected to bilateral I/R, and 3) a group of rats in which adrenal glands were removed 3 days before induction of I/R. As expected, I/R resulted in renal dysfunction and severe tubular injury that was associated with a significant increase in tubular damage markers. In contrast, there was no renal dysfunction or tubular injury in rats that were adrenalectomized before I/R. These effects were demonstrated by normalization of glomerular filtration rate, markers of oxidative stress, and tubular injury markers in adrenalectomized rats. The renoprotection observed was associated with the reestablishment of nitric oxide metabolites, increased endothelial nitric oxide synthase expression and its activating phosphorylation, as well as normalization of Rho-kinase expression and ETA mRNA levels. Our results show that aldosterone plays a central role in the pathogenesis of renal damage induced by I/R and that MR blockade may be a promising strategy that opens a new therapeutic option for preventing acute renal injury.

endothelin; mineralocorticoid receptor; oxidative stress; Rho-kinase

RENAL INJURY INDUCED by ischemia-reperfusion (I/R) is the leading cause of acute kidney injury (AKI) after major cardiovascular surgery, trauma, or kidney transplantation. I/R exerts its deleterious effects by producing renal cell death, renal failure, delayed graft function, and renal graft rejection. AKI may occur in ~5% of hospitalized patients and 30–50% of patients in intensive care units (30, 33). Despite advances in preventive strategies, AKI disease persists with high morbidity and mortality that have not yet to be reduced (26, 29, 33).

AKI is characterized by tubular and vascular injury, as well as by a glomerular podocyte effacement that compromises renal physiological processes (33, 56). This syndrome usually develops after a transient and abrupt drop in renal blood flow (RBF), resulting in the accumulation of nitrogenous and non-nitrogenous waste products. Although reperfusion is essential for the survival of the ischemic tissue, there is evidence that reperfusion itself causes additional cellular damage (57).

The classic role of the adrenal steroid hormone aldosterone is to maintain the homeostasis of salt and potassium by modulating their absorption and reabsorption in the intestine and kidneys, respectively. In the kidney, the actions of aldosterone are mediated through the activation of mineralocorticoid receptors (MR) in the distal nephron. However, it is now known that MR are present in several nonepithelial tissues, and there is growing evidence in humans and animal experimental models of disease that aldosterone is capable of inducing pathophysiological effects in cardiovascular and renal systems (4, 5, 8, 9, 16, 18, 19, 22, 25, 43, 45–48, 53) via its action on MR. Thus aldosterone is currently known as a “profibrotic hormone” (22, 25).

We previously showed that aldosterone plays a key role in the pathophysiology of acute and chronic cyclosporine (CsA) nephrotoxicity (12, 40, 41). In these studies, we noticed that MR blockade with spironolactone reduced not only the structural renal damage associated with CsA but also prevented the renal dysfunction that is known to be due to intense afferent and efferent vasoconstriction (6, 10, 32), suggesting that aldosterone modulates the tone of the renal vasculature. In support of this finding, Arima et al. (2) demonstrated that aldosterone causes vasoconstriction in afferent and efferent arterioles of rabbits. More recently, Gross et al. (17) showed that aldosterone produces dose-dependent contraction of vascular smooth muscle in humans by increasing the phosphorylation of myosin light chains. This effect was inhibited by eplerenone, another MR antagonist. Accordingly, we reasoned that if aldosterone plays an important role in inducing renal vasoconstriction, then MR blockade might be a protective agent in acute renal injury. This hypothesis is strongly supported by our recent observations (37) that prophylactic treatment with spironolactone completely prevented acute tubular necrosis in rats undergoing bilateral renal ischemia. These data, however, raised the question of whether the protective effect of spironolactone was due to MR blockade or to an indirect, unknown effect of spironolactone. Therefore, the present study was designed to evaluate whether the absence of aldosterone has effects similar to the effect of spironolactone in preventing renal injury induced by I/R, and if this is the case, we aim to understand the molecular mechanisms by which aldosterone exerts its deleterious effects on renal function and structure during the process of I/R.

MATERIALS AND METHODS

All experiments involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996) and were approved by our Institutional Animal Care and Use Committee. Twenty-seven male Wistar rats (280–320 g) were included and divided into three groups: 1) rats underwent to sham surgery that were undergone to two sham...
surgeries simulating I/R and adrenalectomy interventions (S), 2 rats subjected to renal bilateral I/R, and 3) rats whose adrenal glands were removed 3 days before being subjected to I/R (Adx+i/R). To prevent hypoglycemia, adrenalectomized rats received subcutaneously 12 μg/kg of dexamethasone daily. This dose has been used previously to maintain physiological levels of glucocorticoids (51). All rats were studied after 24 h of reperfusion.

**Kidney I/R Injury Animal Model**

Rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (30 mg/kg). Animals were placed on a heating pad to maintain constant body temperature and were monitored with a rectal thermometer. A midline abdominal incision was made, and both kidneys were exposed. Renal ischemia was induced by nontraumatic vascular clamps over the pedicles for 20 min. After clamps were released, the incision was closed in two layers with 2-0 sutures. Sham-operated rats underwent anesthesia, a laparotomy, and renal pedicle dissection only.

**Functional Studies**

One hour after renal ischemia, rats were placed in metabolic cages at 22°C with a 12:12-h light-dark cycle and allowed free access to water. Individual 23-h urine samples were collected. Urinary protein excretion was measured by a TCA-turbidimetric method (20), and N-acetyl-β-glucosaminidase (NAG) was measured spectrophotometrically (58). Serum and urine creatinine concentrations were measured with an autoanalyzer (Technicon RA-1000, Bayer, Tarrytown, NY), and renal creatinine clearance was calculated by the standard formula

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C_r = \frac{U \times V}{P}
\]

where \( U \) is the concentration of creatinine in urine, \( V \) is the urine flow rate, and \( P \) is the serum concentration of creatinine. Serum aldosterone was quantitatively determined by radioimmunoassay following the procedures described by the manufacturer (Diasorin, Saluggia, Italy).

Twenty-four hours after renal ischemia, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg) and placed on a homeothermic table. The trachea and femoral arteries were catheterized with polyethylene tubing, PE-240 and PE-50. The rats were maintained under euvolmic conditions by infusing 10 ml/kg body wt of rat plasma during surgery. The mean arterial pressure of each animal was preserved at the mean arterial pressure of each animal. Following perfusion with a phosphate buffer through the femoral catheter, thereby maintaining constant body temperature and were monitored with a rectal thermometer. A midline abdominal incision was made, and both kidneys were exposed. Renal ischemia was induced by nontraumatic vascular clamps over the pedicles for 20 min. After clamps were released, the incision was closed in two layers with 2-0 sutures. Sham-operated rats underwent anesthesia, a laparotomy, and renal pedicle dissection only.

**Histopathological Studies**

At the end of the experiment, the right kidney was removed and quickly frozen for molecular studies, while the left kidney was perfused with a phosphate buffer through the femoral catheter, thereby preserving the mean arterial pressure of each animal. Following excision of the kidney, the perfusate was replaced by a freshly prepared 10% formalin buffer and perfusion was continued until fixation was completed. After appropriate dehydration, kidney slices were embedded in paraffin, sectioned at 5 μm, and stained with an periodic acid-Schiff (PAS) technique. Ten subcortical and juxtamedullary fields were recorded from each kidney slide by using a digital camera incorporated in a Nikon microscope. The affected tubular area was analyzed blindly by an expert nephropathologist. Tubular damage was characterized by a loss of the brush border, lumen dilatation or collapse, and detachment of epithelium from the basement membrane. Digital microphotographs were recorded for each rat to assess, by morphometric analysis, the total tubular area (excluding luminal, interstitial, and glomerular areas) and the total damaged tubular area, delimited by using eclipse net software (magnification, ×400) as we have reported previously (37, 40). The damaged tubular area was expressed as a proportion of the affected tubular area and of the total tubular area.

**Oxidative Stress Determination**

Renal lipoperoxidation. Malondialdehyde (MDA), a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reacting substance (TBARS), as previously reported (37). Briefly, after homogenization of the tissue, the reaction was performed in a 0.8% aqueous solution of thiobarbituric acid in 15% of trichloroacetic acid and heated at 95°C for 45 min. The mixtures were centrifuged at 3,000 g for 15 min. Supernatant absorbance was read at 532 nm. TBARS were quantified using an extinction coefficient of 1.56 × 10^5 M^-1 cm^-1 and expressed as nanomoles of TBARS per milligram of protein. Tissue protein was estimated using the Bradford method.

**Urinary hydrogen peroxide assay**. The amount of hydrogen peroxide (H_2O_2) in urine was determined by using an Amplex Red Hydrogen Peroxide/ Peroxidase Assay Kit (Invitrogen, Eugene, OR) according to manufacturer’s instructions. Briefly, the assay was performed employing a standard curve of H_2O_2 (1–10 μM). A volume of 50 μl of each urine standard was placed in a microplate; 50 μl of Amplex red reagent/HRP were then added and the samples were incubated for 30 min at room temperature, protected from light. The plate was read at 560 nm. The H_2O_2 concentration in the samples was expressed as nanomoles per milliliter. To validate whether this technique could detect H_2O_2 concentration in urine samples accurately, different known concentrations of H_2O_2 were added to rat urine. The H_2O_2 concentrations were 6.53, 5.22, 3.91, and 2.61 μM, and the total H_2O_2 concentrations were detected by the Amplex red kit. Thereafter, endogenous H_2O_2 in each sample was subtracted to get the percentage of H_2O_2 recovery. The average H_2O_2 recovery percentages in the samples were 96.49 ± 4.03, 102.80 ± 3.23, 100.83 ± 3.05, and 95.56 ± 3.5%, respectively. The average variation coefficient was <5%.

**Molecular Studies**

**RNA isolation and real-time RT-PCR**. The renal cortex was isolated from both kidneys and snap frozen in liquid nitrogen. Total RNA was isolated from each kidney following the TRIZol method (Invitrogen, Carlsbad, CA) and checked for integrity by 1% agarose gel electrophoresis. To avoid DNA contamination, all total RNA samples were treated with DNAAse (DNAase I, Invitrogen). RT was carried out with 2.5 μg of total RNA using 200 U of the Moloney murine leukemia virus reverse transcriptase (Invitrogen). The mRNA levels of endothelial nitric oxide synthase (eNOS), Rho-kinase, prepro-endothelin, and endothelin A and B receptors (ET_A and ET_B) were quantified by real-time PCR on the ABI Prism 7300 Sequence Detection System (TaQMan, ABI, Foster City, CA). Primers and probes were ordered as kits: Rn02132634_s1 for eNOS, Mm00485745_m1 for Rho-kinase, Rn00561129_m1 for prepro-endothelin, and Rn00561137_m1 for the ET_A receptor and Rn00597703_m1 for the ET_B receptor (Assays-on-Demand, ABI). As an endogenous control, we used eukaryotic 18S rRNA (predesigned assay reagent, external run, ABI). The relative quantification of eNOS, Rho-kinase, prepro-endothelin, ET_A and ET_B gene expression was performed using the comparative CT method (35).

**Western blot analysis**. Renal proteins were isolated by homogenization and used for immunoblot analysis with a rabbit eNOS antibody (Cell Signaling Technology), phospho-eNOS Thr495 antibody (Cell Signaling Technology, Danvers, MA), phospho-eNOS Ser1177 antibody (Cell Signaling Technology), or goat Rho-kinase antibody (Santa Cruz Biotechnology, Santa Cruz, CA); each one was used at 1:500 Afterward, membranes for eNOS and phospho-ENOS levels detection were incubated with a secondary antibody, HRP-conjugated rat anti-rabbit IgG (1:2,500, Alpha Diagnostics, San Antonio, TX), whereas for Rho-kinase detection donkey anti-
goat IgG-HRP (Santa Cruz Biotechnology) was used. To control protein loading and transfer, all membranes were simultaneously probed with an actin antibody (1:2,500) and a secondary antibody, donkey anti-goat IgG-HRP (Santa Cruz Biotechnology). Proteins were detected with an enhanced chemiluminescence kit (Amer-sham) and autoradiography, following the manufacturer’s recommendations. All Western blot analyses were performed within the linear range of protein loads and antibody use. The bands were scanned for densitometric analysis.

Biochemical Studies

Antioxidant enzyme activity. Renal tissue was homogenized in 50 mM potassium phosphate, 0.1% Triton (pH = 7.0). Crude homogenates were centrifuged at 15,300 rpm for 30 min at 4°C and used to assess antioxidant enzyme activities. Total protein concentration was measured by Lowry’s method (36).

Catalase activity assay. Renal catalase activity was determined according to the Aebi method (1). This method is based on measuring the decomposition of H₂O₂ by catalase at an optical density of 240 nm at times 0 and 15 s (first-order kinetics) given the equation \( k = (1/\Delta t) (2.3 \times \log A_1/A_2) \), where \( k \) is the first-order reaction rate constant, and \( \Delta t \) is the time interval of the decrease in H₂O₂. An enzyme activity unit was defined as the degradation of 1 μmol of H₂O₂ per second per milligram of tissue protein, and the enzyme activity was expressed as \( k \) per milligram protein when assayed at 25°C by a method based on the disappearance of H₂O₂ from a solution containing 30 mM H₂O₂ in 10 mM potassium phosphate buffer, pH 7.0 (output read at 240 nm). The results are expressed in \( k \) per milligram protein.

Glutathione peroxidase activity. Glutathione peroxidase (GPx) activity was indirectly detected by a method previously described by Lawrence and Burk (34). GPx requires GSSG, which is regenerated by glutathione reductase using GSH, a process that consumes NADPH. The activity of GPxs was defined as micromoles NADPH oxidized per minute, taking into account that the millimolar absorption coefficient for NADPH at 340 nm is 6.22 1·mmol⁻¹·cm⁻¹. The results are expressed as units per milligram protein.

SOD activity. SOD activity was measured through the inhibition of nitrotetrazolium blue (NBT) reduction by O₂ generated through the xanthine-xanthine oxidase system as previously reported (34). One SOD activity unit was defined as the amount of enzyme promoting 50% inhibition of NBT reduction in 1 ml reaction solution/mg tissue protein. Results are expressed as units per milligram protein.

Fig. 1. Functional parameters in the sham, ischemia-reperfusion (I/R), and adrenalectomized (Adx) rats before I/R (Adx-I/R) groups; \( n = 9 \). A: rats with I/R exhibited a significant elevation of serum aldosterone that was completely suppressed in adrenalectomized rats. Renal failure in the I/R group was evidenced by a significant reduction of renal blood flow (RBF; C) and renal creatinine clearance (D) without changes in mean arterial pressure (MAP; B). Renal dysfunction was completely prevented in the rats that were adrenalectomized previous to I/R. *\( P < 0.05 \) vs. all studied groups.
Statistical Analysis

Results are presented as means ± SE. The significance of the differences among groups was tested by ANOVA using Bonferroni’s correction for multiple comparisons. Statistical significance was defined as being a P value <0.05.

RESULTS

We first verified that the adrenal glands were removed properly by measuring the levels of serum aldosterone. Because renal hypoperfusion is one of the most important mechanisms to induce aldosterone secretion, the I/R group exhibited a significant increase in serum aldosterone levels of more than fivefold that of the sham-treatment group (1,994.9 ± 134.5 vs. 356 ± 77.1 pg/ml) as is shown in Fig. 1A. As expected, serum aldosterone was nearly absent in adrenalectomized rats (27.2 ± 6.8 pg/ml), confirming that adrenal gland removal was carried out in a correct way and that the animals who were subjected to I/R had minimal aldosterone levels.

After 24-h of reperfusion, the groups of rats that were subjected to I/R showed no changes in mean arterial blood pressure compared with rats receiving a sham operation, as shown in Fig. 1B. However, renal I/R produced renal dysfunction characterized by an increase in serum creatinine levels (0.3 ± 0.1 to 0.8 ± 0.2 mg/dl; P < 0.01), and by a significant reduction in RBF by 46% (Fig. 1C). As a result, creatinine clearance was reduced from 1.5 ± 0.2 to 0.7 ± 0.1 ml/min, as outlined in Fig. 1D (P < 0.01). In contrast, renal dysfunction was not observed in rats that were adrenalectomized before renal I/R. Creatinine clearance of control and Adx + I/R group was 1.5 ± 0.2 and 1.3 ± 0.2 ml/min, respectively (Fig. 1D; P = NS); thus serum creatinine levels between both groups were similar (0.3 ± 0.1 and 0.5 ± 0.1 mg/dl, respectively, P = NS). Similarly, the removal of the adrenal glands in rats subjected to I/R prevented hypoperfusion, as can be seen in Fig. 1C. To rule out that adrenalectomy itself did not affect basal renal function parameters, we included an additional group in which adrenal glands were removed 3 days before sham surgery was performed. After 24 h, this group exhibited a mean value of mean arterial pressure of 118 ± 5.6 mmHg, the RBF was 6.3 ± 0.3 ml/min, and the creatinine clearance was 1.35 ± 0.1 ml/min. Thus adrenalectomy had no effect on renal function.

Histopathological studies revealed that the I/R group developed acute tubular necrosis, characterized by severe loss of the brush border, tubular dilation, tubular collapse, and tubular basement membrane detachment (Fig. 2A, top left). All these lesions were absent in the adrenalectomized rats, as shown in the representative bottom left image (Fig. 2B). This observation was confirmed by quantitative morphometric analysis, in which the percentage of tubular damaged area was determined (Fig. 2C) and by assessment of the classic tubular injury markers (Fig. 2D–F).

Fig. 2. A and B: subcortical histological microphotographs of kidneys stained with periodic acid-Schiff from I/R and Adx + I/R groups, respectively. A: representative microphotograph in high power (×400) of a kidney section from an I/R rat showing extensive tubular injury characterized by detachment of tubular epithelial cells from the basement membrane, tubular dilation, loss of the brush border, and flattened epithelial cells, as well as the presence of hyaline cast. B: these lesions were practically absent in adrenalectomized rats before I/R. C: morphometric quantification of affected tubular area. D: urinary N-acetyl-β-glucosaminidase (NAG) excretion. E: urinary protein excretion. F: renal mRNA levels of kidney injury molecule. *P < 0.05 vs. all studied groups; n = 9.
markers as detailed in Fig. 2, D and E. In this way, the percentage of tubular affected area in the I/R group was 66.2 ± 0.7%, while it dropped in the adrenalectomized group to 6.1 ± 2.0% (P < 0.0001). Protein and NAG urinary excretion increased five and three times, respectively, in the group with renal I/R. In this group, renal Kim-1 mRNA levels increased 500-fold compared with rats in the sham operation group. In contrast, the increase in tubular injury markers for rats in which the adrenal glands were removed was significantly attenuated. These results clearly show that the absence of aldosterone prevented I/R-induced kidney damage and strongly suggest that aldosterone plays a key role as a mediator of the structural and functional impairment of the kidneys in this model of renal injury.

Because there is evidence that part of the renal injury induced by I/R is due to endothelial dysfunction and vascular damage (39), we assessed oxidative stress and indirectly measured nitric oxide (NO) production. Oxidative stress was evaluated through quantifying the amount of MDA, a marker of renal tissue lipoperoxidation, and by determining the excretion of hydrogen peroxide in urine (UH₂O₂V). As Fig. 3 shows, I/R produced a significant increase in renal lipoperoxidation and urinary H₂O₂ excretion (UH₂O₂V) compared with the control group. MDA levels were 0.30 ± 0.04 vs. 0.07 ± 0.01 mmol/mg protein (P < 0.05) and UH₂O₂V were 38.0 ± 11.8 vs. 3.9 ± 2.2 nmol/min (P < 0.01). These abnormalities in MDA and UH₂O₂V were not seen in adrenalectomized rats, as values in these rats were similar to those observed in control animals (0.17 ± 0.03 mmol/mg protein and 6.0 ± 4.4 nmol/min, respectively), suggesting that aldosterone participates in producing oxidative stress during the I/R phenomenon. The oxidative stress observed in the I/R group and its prevention by adrenalectomy was not associated with changes in renal antioxidant enzyme activity, as shown in Fig. 4, or mRNA expression levels (data not shown).

We also evaluated the urinary excretion of stable metabolites of NO synthesis, nitrites and nitrates (UNO₂/NO₃V). As we previously reported (37), a significant decrease by 50% in UNO₂/NO₃V was observed in rats subjected to I/R. Interestingly, adrenal gland removal completely prevented the decrease in UNO₂/NO₃V, as shown in Fig. 5A. To investigate how the adrenalectomy prevented the reduction of UNO₂/NO₃V, the
Fig. 5. A: renal injury induced by I/R was associated with a significant reduction in urinary nitric oxide (NO) metabolites (UNO$_2$/NO$_3$/V), an effect that was prevented by the removal of the adrenal glands. B–F: effect of I/R and adrenalectomy on endothelial nitric oxide synthase (eNOS) and eNOS phosphorylation that was evaluated by Western blot analysis using specific eNOS and phospho-eNOS antibodies in pooled kidney proteins from 6 rats. Renal eNOS expression and phosphorylation were not altered by I/R injury. On the contrary, eNOS protein expression and the amount of phospho-eNOS Ser1178 were increased in the rats that were adrenalectomized before I/R. *P < 0.05 vs. all compared groups.
levels of expression of eNOS and its phosphorylation state were evaluated. As we have shown before (37), eNOS mRNA levels were not affected by I/R, as is depicted in Fig. 5B, and similar results were found when eNOS protein levels were measured (Fig. 5, C and D). In contrast, levels of eNOS protein in the kidney were significantly increased in rats that were previously adrenalectomized (Fig. 5D). This effect was associated with a more than twofold increase in the activating phosphorylation of eNOS (Ser1178), as shown in Fig. 5E, whereas the inactivating phosphorylation of eNOS (Thr494) was not modified by either I/R or adrenalectomy (Fig. 5F). These findings for rats in which adrenal glands were removed are similar to those observed during prevention of I/R injury by spironolactone (37). Because it has been reported that aldosterone produces endothelial injury by reducing the expression of glucose-6 phosphate dehydrogenase (G-6PD) (61), we assessed renal mRNA levels of this enzyme. As is shown in Fig. 6, G-6PD mRNA levels were upregulated in the I/R group, an effect that was not modified by adrenalectomy. These results suggest that G-6PD might be regulated by other factors activated during an I/R process that are independent of aldosterone.

Since it is known that Rho-kinase is involved in signaling pathways that mediate vasoconstriction, another potential mechanism that was explored to determine how aldosterone induces renal injury during I/R was the expression level of Rho-kinase. As shown in Fig. 7A, in the kidneys of rats with ischemia, mRNA levels of Rho-kinase were significantly higher. Of note, in the group that was previously adrenalectomized, Rho-kinase mRNA levels were similar to that in the control group. Similar results for Rho-kinase expression were observed at the protein level by Western blot analysis. As is shown in Fig. 7B, Rho-kinase increased by twofold in the I/R group, but this overexpression was not seen in the animals in which adrenal glands were removed before the I/R insult.

Similarly, it is known that endothelin is a potent vasoconstrictor in renal arteries; thus we also assessed the mRNA levels of prepro-endothelin and its receptors. As is shown in Fig. 8, prepro-endothelin expression levels significantly increased in both the I/R and adrenalectomized groups. However, the I/R group demonstrated a greater than twofold increase in ETA and ETB mRNA levels, while the absence of aldosterone prevented an increase in the ETA receptor, known to be the mediator of endothelin-induced vasoconstriction, and enhanced to an even greater extent the mRNA levels of the ETB receptor, a receptor known to mediate renal vasodilatation (28).
Inflammation, cytoskeletal breakdown, sublethal cell injury, apoptosis, loss of tubular polarity, tubular obstruction, interstitial cells. Occurring at the same time, the tubular component encompasses loss of tubular polarity, tubular obstruction, interstitial inflammation, cytoskeletal breakdown, sublethal cell injury, apoptosis, and tubular necrosis. The mechanisms responsible for these tubular alterations include hypoxia, ATP depletion, increased concentrations of reactive oxygen species (ROS), intracellular acidosis, elevated cytosolic calcium concentrations, increased activity of phospholipases, and proteases released from the tubular cell brush border (for a review, see Ref. 33).

In this study, we found that both components, vascular and tubular, were profoundly affected by 20 min of bilateral ischemia and 24 h of reperfusion in a rat model. Accordingly, RBF and glomerular filtration rate were significantly reduced; we also found evidence of severe tubular injury. We have previously shown that spironolactone administration prevents the renal vasoconstriction characteristic of acute and chronic CsA-induced nephrotoxicity in humans, as well as in an animal model of renal I/R injury (12, 37, 40, 41), suggesting that aldosterone plays a key role in producing renal vasoconstriction in these models of ischemic damage. However, in our previous studies, the mechanism for spironolactone protection was not defined, since we reasoned that the renoprotective effect of spironolactone could be mediated by either MR blockade or by other unknown properties of spironolactone. To begin to understand the protective effect of spironolactone, in the present study we assessed the consequence of adrenal gland removal before the rats were exposed to the I/R insult. Adrenalectomy not only suppresses the normal circulating aldosterone levels but also completely prevents the aldosterone secretion induced by renal hypoperfusion. Thus, instead of blocking the MR receptor (as with spironolactone), here we tested the absence of aldosterone secretion.

As we have seen previously in spironolactone-treated rats (37), adrenalectomy completely prevented renal dysfunction and preserved tubular structure in the Adx + I/R group. Creatinine clearance, RBF, and renal histology in rats that underwent adrenalectomy before I/R were similar to sham operation rats. The renoprotection induced by adrenalectomy was also evidenced by the reduction in tubular injury markers, which included NAG, proteinuria, and renal cortex Kim-1 mRNA levels. In this regard, we have shown that Kim-1 expression is a sensitive tool for detecting tubular damage in different models of renal injury, including I/R (54, 55).

It has been shown that AKI is associated with increased renal interstitial cathecholamine levels and that blockade of the α1-adrenoceptor is protective against ischemic injury in rats (14). In this regard, protection conferred by adrenalectomy could be due, at least in part, by a reduction in adrenaline levels. However, our previous studies (37) clearly showed that renal injury induced by I/R is completely prevented by blocking the MR with spironolactone. Thus we believe that the protection observed in adrenalectomized animals is mainly mediated by a reduction in aldosterone levels, rather than adrenaline levels.

I/R leads to renal vascular dysfunction with subsequent impairment of RBF, further increasing initial renal injury (39). The reduction in RBF is mainly mediated by an imbalance of vasoactive substance release, as was previously mentioned. Wang et al. (59) have shown that aldosterone increases endothelin levels. Here, we observed that prepro-endothelin mRNA expression levels were similarly increased in ischemic and adrenalectomized rats. However, endothelin receptor expression was differentially modulated. Two kinds of endothelin receptors are known to be expressed in the kidney: ETα and ETβ.

**DISCUSSION**

The consequence of I/R that often occurs during hypovolemic shock, transplantation, or major cardiovascular surgery is often AKI. Vascular and tubular defects take place after ischemia and during the reperfusion process. The vascular component includes intrarenal vasoconstriction that leads to a glomerular filtration rate reduction, together with vascular congestion in the outer medulla and activation of tubuloglomerular feedback. The mechanisms implicated in this response are as follows: increased release of vasoconstrictor factors (mainly endothelin, adenosine, and angiotensin II); decreased production of vasodilators (such as NO, prostaglandin, acetylcholine, and bradykinin); and increased structural damage in endothelial and vascular smooth muscle cells. Occurring at the same time, the tubular component encompasses loss of tubular polarity, tubular obstruction, interstitial inflammation, cytoskeletal breakdown, sublethal cell injury, apoptosis, and tubular necrosis. The mechanisms responsible for these tubular alterations include hypoxia, ATP depletion, increased concentrations of reactive oxygen species (ROS), intracellular acidosis, elevated cytosolic calcium concentrations, increased activity of phospholipases, and proteases released from the tubular cell brush border (for a review, see Ref. 33).

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It has been shown that AKI is associated with increased renal interstitial cathecholamine levels and that blockade of the α1-adrenoceptor is protective against ischemic injury in rats (14). In this regard, protection conferred by adrenalectomy could be due, at least in part, by a reduction in adrenaline levels. However, our previous studies (37) clearly showed that renal injury induced by I/R is completely prevented by blocking the MR with spironolactone. Thus we believe that the protection observed in adrenalectomized animals is mainly mediated by a reduction in aldosterone levels, rather than adrenaline levels.

I/R leads to renal vascular dysfunction with subsequent impairment of RBF, further increasing initial renal injury (39). The reduction in RBF is mainly mediated by an imbalance of vasoactive substance release, as was previously mentioned. Wang et al. (59) have shown that aldosterone increases endothelin levels. Here, we observed that prepro-endothelin mRNA expression levels were similarly increased in ischemic and adrenalectomized rats. However, endothelin receptor expression was differentially modulated. Two kinds of endothelin receptors are known to be expressed in the kidney: ETα and
ETB. These receptors mediate vasoconstriction or vasodilatation, respectively, induced by endothelin. Consistent with renoprotection induced by adrenalectomy, while mRNA levels of ETα and ETβ receptors were upregulated in the I/R group. In adrenalectomized rats, however, ETα receptor expression was not increased, whereas expression of ETβ receptors was enhanced, compared with the I/R group (Fig. 8). Similar findings were also observed in chronic CsA nephropathy, in which the aldosterone effect was blocked by spironolactone (41). These results suggest that in the presence of aldosterone, endothelin mediates renal vasoconstriction through ETα receptors, with the concomitant compensatory elevation of ETβ receptors. In contrast, in the absence of aldosterone, although endothelin is also increased during I/R, it is probably promoting vasodilatation, rather than vasoconstriction, due to the proportional increase in ETβ over ETα receptor expression.

Recent advances in vascular cell biology have elucidated the substantial involvement of the small GTPase Rho and its downstream effector Rho-kinase in promoting vascular smooth muscle cell contraction by inactivating myosin phosphatase and subsequently increasing myosin light chain phosphorylation (50). Specifically for AKI, it has been observed that during renal I/R, Rho-kinase mRNA and protein levels increase (7). In addition, renal injury induced by long-term aldosterone administration has been associated with increases in myosin phosphatase target subunit-1, a marker of Rho-kinase activity (52). These studies suggest that Rho-kinase might be involved in the renal vasoconstriction observed after ischemia. Thus the Rho/Rho-kinase pathway has recently attracted much attention in various research fields for its participation in vascular tone regulation and fibrosis development. In fact, in our study we detected that in kidneys isolated from I/R rats that exhibited considerable increases in aldosterone, Rho-kinase mRNA and protein levels were significantly upregulated. Interestingly, this effect was completely prevented by adrenalectomy. Consistent with this observation, Rho-kinase mRNA upregulation has been observed in hypertensive Dahl salt-sensitive rats and the expression levels of this kinase were restored by eplerenone (52).

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