Renal ischemia-reperfusion injury is prevented by the mineralocorticoid receptor blocker spironolactone

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Renal ischemia-reperfusion injury is prevented by the mineralocorticoid receptor blocker spironolactone. Am J Physiol Renal Physiol 293: F78–F86, 2007. First published March 20, 2007; doi:10.1152/ajprenal.00077.2007.—Renal ischemia and reperfusion (I/R) injury is the major cause of acute renal failure and may also be involved in the development and progression of some forms of chronic kidney disease. We previously showed that a mineralocorticoid receptor (MR) blockade prevents renal vasoconstriction induced by cyclosporine that leads to acute and chronic renal failure (Feria I, Pichardo I, Juárez P, Ramírez V, González MA, Uribe N, García-Torres R, López-Casillas F, Gamba G, Bobadilla NA. Kidney Int 63: 43–52, 2003; Perez-Rojas JM, Derive S, Blanco IA, Cruz C, Martínez de la Maza L, Gamba G, Bobadilla NA. Am J Physiol Renal Physiol 289: F1020–F1030, 2005). Thus we investigated whether spironolactone administration prevents the functional and structural damage induced by renal ischemia-reperfusion (I/R). Five groups were studied: sham-operated animals, rats that underwent 20 min of ischemia and 24 h of reperfusion, and three groups that received spironolactone 1, 2, or 3 days before I/R, respectively. Renal I/R produced significant renal dysfunction and tubular damage. Spironolactone administration completely prevented a decrease in renal blood flow, the development of acute renal failure, and tubular apoptosis. The protection conferred by spironolactone was characterized by decreasing oxidative stress, as evidenced by a reduction in kidney lipoperoxidation, increasing expression of antioxidant enzymes, and restoration of urinary NO2/NO3 excretion. Endothelial nitric oxide synthase expression was upregulated by a mineralocorticoid receptor blockade in I/R groups; in addition, an increase in activating phosphorylation of this enzyme at residue S1177 and a decrease in inactivating phosphorylation at T497 were observed. In conclusion, our study shows that spironolactone administration prevents the renal injury induced by I/R, suggesting that aldosterone plays a central role in this model of renal injury.

endothelial nitric oxide synthase; apoptosis; lipoperoxidation

RENAL ISCHEMIA-REPERFUSION (I/R) injury is the major cause of acute renal failure in both native and transplanted kidneys (22). Ischemic acute renal failure is a syndrome that develops following a transient drop in total or regional blood flow to the kidney. Although reperfusion is essential for the survival of ischemic tissue, there is evidence that reperfusion itself causes additional cellular injury (48). The mechanisms of renal I/R injury involve both vascular and tubular factors, but despite advances in preventive strategies, this disease continues to be associated with significant morbidity and mortality (21) and there is no successful specific therapy except for supportive care (10).

Recent studies in humans and experimental models have shown that aldosterone plays a pivotal role in the pathophysiology of cardiovascular and renal injury. In this regard, clinical trials have evidenced that mineralocorticoid receptor (MR) blockade improves the survival of patients with chronic heart disease (29, 30, 33, 34) and chronic renal failure (4, 5). The protective effect of MR blockade is associated with decreased fibrosis and vascular inflammation, suggesting that aldosterone is a profibrotic hormone (16, 19). In addition, the effectiveness of MR antagonism in ameliorating glomerular and/or tubulo-interstitial injury has also been documented in several models of nephropathy, including spontaneously hypertensive stroke-prone rats (36, 37), angiotensin II- and nitric oxide synthase inhibitor-treated rats (38), aldosterone-treated rats (15), diabetic nephropathy type 1 and 2 (13), and in a model of unilateral ureteral obstruction (44). We previously observed that aldosterone also plays an important role in nephrotoxicity induced by the immunosuppressant cyclosporine A (CsA), an agent that is extensively used for prevention of allograft rejection (8, 31, 32). In these studies, we observed that in chronic nephropathy an MR blockade reduced structural injury (32) and helped to avoid the progression of renal damage in a model of preexisting chronic CsA nephrotoxicity (31) by mechanisms that involved the reduction of TGF-β expression, lipoperoxidation, and cell death by apoptosis. Interestingly, in the course of these studies, we noticed that an MR blockade prevents the well-known effect of CsA-inducing renal vasoconstriction (2, 7, 8). CsA administration in rats for 7 days was associated with a reduction in renal blood flow by 50%. This reduction was completely prevented by spironolactone (32), suggesting that aldosterone modulates renal vascular tone in this model. In support of these findings, Gros et al. (12) have recently showed that acute aldosterone exposure induced a dose-dependent vasoconstriction through myosin light chain phosphorylation in clonal adult human vascular smooth muscle cells. This effect was prevented by spironolactone, suggesting that aldosterone-mediated vasoconstriction may represent an important pathophysiological mechanism of vascular disease. Thus we reasoned that if aldosterone plays a role in renal vasoconstriction, then spironolactone could be protective against acute I/R injury.
MATERIALS AND METHODS

Thirty male Wistar rats (200–300 g) were included and divided into five groups: sham-operated (sham), rats subjected to ischemia-reperfusion (I/R), and three groups of rats that were treated during 1, 2 or 3 days with spironolactone (20 mg/kg by gastric gavage) before I/R was performed (Sp1, Sp2, and Sp3, respectively). This is the most commonly used dose of spironolactone in rats (8, 9, 13, 25, 35, 37, 47).

Kidney I/R injury animal model. Rats were anesthetized by intraperitoneal injection with pentobarbital sodium (30 mg/kg). Animals were placed on a heating pad to maintain a constant temperature and 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 animals underwent anesthesia, laparotomy, and renal pedicle dissection only. All animal procedures were followed in accordance with our institutional guidelines for animal care.

Functional parameters. Two hours 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 24-h urine samples were collected. Urinary protein excretion was measured by a TCA turbidimetric method (14) and N-acetyl-β-glucosaminidase (NAG) was measured spectrophotometrically (49). 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 C = (U × V)/P, where U is the concentration in urine, V is the urine flow rate, and P is the serum concentration. Serum aldosteron was quantitatively determined by radioimmunoassay following the procedures described by the manufacturer (DiaSorin, Saluggia, Italy).

Twenty-four hours after renal ischemia-reperfusion, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (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 euolemic conditions by infusing 10 ml/kg body wt of isotonic rat plasma during surgery. The mean arterial pressure was monitored with a pressure transducer (model p23 db, Gould) and recorded on a polygraph (Grass Instruments, Quincy, MA). Via a midline abdominal incision, the left renal artery was exposed. An ultrasound transit-time flow probe (1RB, Transonic, Ithaca, NY) was placed around the left renal artery and filled with ultrasonic coupling gel (HR Lubricating Jelly, Carter-Wallace, New York, NY) for recording the renal blood flow.

Histopathological studies. At the end of the experiment, the right kidney was removed and quickly frozen for molecular studies and the left kidney was perfused through the femoral catheter with a phosphate buffer, thereby preserving the mean arterial pressure of each animal. Following blanching 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 4-μm and stained via the periodic acid-Schiff technique. Ten subcortical and 10 juxtamedullary fields (magnification ×400) were scanned for densitometric analysis. The affected tubular area was expressed as a proportion of the affected tubular area and total tubular area.

Terminal transferase-dUTP-nick-end labeling assay. Apoptosis in kidney sections was determined by terminal transferase-dUTP-nick-end labeling (TUNEL) assay using an ApopTag in situ apoptosis detection kit (S7101, Chemicon International, Temecula, CA). Slides were prepared by following the procedures previously described (50). A minimum of 10 subcortical and 10 juxtamedullary fields (magnification ×400) per kidney were evaluated in all kidney tissues, and the images were recorded and analyzed blindly. Only tubular cells that contained TUNEL-positive nuclei with the characteristic morphology of apoptosis, including nuclear fragmentation and nuclear condensation, were quantified. TUNEL-positive cells were counted, and the results were expressed as the number of TUNEL-positive nuclei per square millimeter.

Renal liperoxidation. Malondialdehyde (MDA), a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances (TBARS) as previously reported (43). Briefly, after homogenization of the tissue, the reaction was performed in an 0.8% aqueous solution of thiobarbituric acid in 15% TCA and heated at 95°C for 45 min, and 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/cm and expressed as nanomoles of TBARS per milligram of protein. The tissue protein was estimated using the Bradford method.

RNA isolation and real-time PCR. Total RNA was isolated from each kidney following the guanidine isothiocyanate-cesium chloride method (40) and checked for integrity by 1% agarose gel electrophoresis. Reverse transcription was carried out using 2.5 μg of total RNA from each rat at 37°C for 60 min using 200 U of the Moloney murine leukemia virus reverse transcriptase (Invitrogen). The mRNA levels of SOD, GPx, catalase, and procaspase-3 were quantified by real-time PCR with the ABI Prism 7300 Sequence Detection System (TagMan, Applied Biosystems, Foster City, CA). FAM or VIC dye-labeled probes were selected from the Applied Biosystems Assays-on-Demand ABI product line and were specifically used to detect and quantify cDNA sequences without detecting genomic DNA. Primers and probes for SOD, GPx, catalase, and procaspase-3 were ordered as kits: Rn00589772_m1, Rn00577994_g1, Rn00560930_m1, and Rn00563902_m1 (Assays-on-Demand, ABI). As endogenous control, we used eukaryotic 18S rRNA (predesigned assay reagent, ABI, external control), and probes for SOD, GPx, catalase, and procaspase-3 were ordered as kits: Rn00589772_m1, Rn00577994_g1, Rn00560930_m1, and Rn00563902_m1 (Assays-on-Demand, ABI). As endogenous control, we used eukaryotic 18S rRNA (predesigned assay reagent, ABI, external control) to correct for potential variations in RNA loading or the efficiency of the amplification reaction. The relative quantification of SOD, GPx, catalase, and procaspase-3 gene expression was performed using the comparative CT method (24).

Western blot analysis. Renal proteins were isolated by homogenization and used for immunoblot analysis with rabbit endothelial nitric oxide synthase (eNOS) antibody, phospho-eNOS T495 antibody, or phospho-eNOS S1177 antibody, all used at 1:500 (Cell Signaling Technology). Afterward, membranes were incubated with a secondary antibody, horseradish peroxidase-conjugated anti-rabbit IgG (1:2,500, Alpha Diagnostics, San Antonio, TX). To control protein loading and transfer, all membranes were simultaneously probed with an actin antibody (1:2,500) and secondary antibody donkey anti-goat IgG- horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were detected with an enhanced chemiluminescence kit (Amersham) 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.

Statistical analysis. Results are presented as means ± SE. Significance of the differences among groups was tested by ANOVA using Bonferroni’s correction for multiple comparisons. All comparisons passed the normality test. Statistical significance was defined when the P value was <0.05.

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RESULTS

We first investigated whether a prophylactic MR blockade prevented renal dysfunction and structural injury induced by renal I/R in the rat. As shown in Fig. 1, after 24 h of renal reperfusion, rats which underwent renal ischemia developed renal dysfunction that was evidenced by an increase of serum creatinine from 0.56 ± 0.06 to 1.64 ± 0.06 mg/dl and the concomitant reduction of creatinine clearance from 1.4 ± 0.2 to 0.4 ± 0.1 ml/min (Fig. 1, A and B). Renal impairment was, in part, related to renal plasma flow reduction by 33%, without changes in mean arterial pressure (Fig. 1, C and D). In contrast, in the three groups that were pretreated with the MR blocker spironolactone for 1, 2, or 3 days before renal I/R (Sp1, Sp2, and Sp3 groups), the fall in renal plasma flow was completely prevented (Fig. 1C), which was associated with normalization of serum creatinine (Sp1: 0.88 ± 0.2 mg/dl, Sp2: 0.66 ± 0.1 mg/dl, and Sp3: 0.60 ± 0.04 mg/dl) (Fig. 1A) and the concomitant reestablishment of renal function, as shown by the values of creatinine clearance (Fig. 1B).

Light microscopic studies revealed that renal I/R produced severe tubular damage characterized by a loss of brush border, lumen dilatation or collapse, and cellular detachment from tubular basement membranes observed in both renal cortex (Fig. 2, A and E; low and high power, respectively) and outer medulla (not shown). All these lesions were practically absent in rats exposed to spironolactone previous to renal I/R (Fig. 2, B–H) and were quantitatively confirmed by morphometric analysis of the percentage of injured tubular areas. As a result, the percentage of affected tubular areas in the I/R group was 66.2 ± 0.7 compared with 6.1 ± 1.4, 9.1 ± 2.5, and 6.6 ± 2.0% in the Sp1, Sp2, and Sp3 groups, respectively (Fig. 2I). An assessment of classic markers of tubular injury supported these observations. Proteinuria and NAG excretion increased five- and threefold, respectively, in the I/R group. Accordingly, in rats pretreated with spironolactone, the levels of these tubular injury markers were similar to those found in sham-operated rats and significantly different from the untreated I/R group (Fig. 2, J and K). Figure 3A shows that animals subjected to renal I/R presented a significant elevation of serum aldosterone levels by more than fivefold compared with sham-operated rats. Aldosterone levels were not different among I/R groups. As shown in Fig. 3B, an MR blockade in this model was not associated with an increase in serum potassium levels.

We observed a significant fall in the amount of urinary NO2/NO3 excretion by one-half in I/R untreated rats, suggesting that nitric oxide (NO) deficiency contributes not only to reduce renal plasma flow but also to extend renal injury. Also noteworthy was the fact that spironolactone completely prevented the fall in urinary NO2/NO3 excretion, as shown Fig. 4A. Thus we analyzed the expression levels and phosphorylation state of eNOS. The eNOS expression levels and phosphorylation in S1177 and T497 residues were not affected by I/R, as...
shown in Fig. 4, B–D. Kidney eNOS protein levels were significantly increased in rats that received the prophylactic treatment with spironolactone (Fig. 4B). In addition, spironolactone treatment was associated with an increase in activating eNOS S1177 phosphorylation (Fig. 4C) and a decrease in inactivating eNOS T497 phosphorylation (Fig. 4D).

We thus reasoned that spironolactone might also be associated with the prevention of a reperfusion-induced increase in oxidative stress and apoptosis. Therefore, kidney lipoperoxidation and the mRNA levels of antioxidant enzymes were evaluated. Tubular ischemic injury induced by I/R was associated with a significant increase in kidney lipoperoxidation by threefold, which was prevented by the prophylactic treatment with an MR blocker, since the first day of pretreatment (Fig. 5A). The reduction of lipoperoxidation observed in the spironolactone-treated groups was accompanied by a significant increase in SOD and glutathione peroxidase as antioxidant enzymes, while the catalase mRNA levels did not change (Fig. 5, B–D).

In situ labeling of cell nuclei by the TUNEL method showed that I/R produced a significant increase in apoptosis measured by positive nuclei stain per square milliliter that was more evident in juxtamedullary areas than in subcortical sections, as shown in Fig. 6, A and B. These observations were

Fig. 2. A–H: subcortical histological sections of kidneys stained with periodic acid-Schiff (PAS) from groups studied as stated. Lower-power (×100; A–D) and high-power (×400; E–H) microphotographs are shown. A and E are representative microphotographs of a kidney section from an I/R untreated rat. Arrowheads in E indicate detachment from basement membrane of tubular epithelial cells; yellow arrow indicates tubular dilation, loss of brush border, and flattened epithelial cells, and HC indicates the presence of hyaline cast. These lesions were practically absent in I/R rats treated with spironolactone from 1–3 days (B–H).

I: morphometric quantification of affected tubular area. J: urinary protein excretion levels. K: urinary N-acetyl-β-glucosaminidase (NAG) excretion. Error bars represent SE. **P < 0.05 vs. all studied groups.
confirmed by the quantification of positive nuclei per square milliliter, graphically expressed in Fig. 6I, showing >100 and 300 positive nuclei/mm² in subcortical and juxtamedullary kidney sections, respectively, of rats that were subjected to renal I/R. Spironolactone pretreatment for 1 day before renal I/R reduced the number of positive nuclei in both areas (Fig. 6B and C and I and J). Nevertheless, apoptosis was completely prevented when an MR blockade started 2 or 3 days before I/R (Fig. 6C–J). In accordance with these observations, renal I/R injury produced a significant increase in pro-caspase-3 mRNA levels and this effect was reversed by the pretreatment with a MR blockade administered starting on the first day before I/R (Fig. 6K).

Fig. 3. Effect of I/R and mineralocorticoid (MR) blockade treatment on serum aldosterone and potassium levels. A: all rats that were subjected to I/R presented a marked elevation of serum aldosterone levels compared with the sham-operated group. B: neither renal I/R nor spironolactone treatment modified serum potassium levels. Error bars represent SE. *P < 0.05 vs. sham-operated rats.

Fig. 4. Renoprotective mechanisms of MR blockade in renal injury induced by I/R. A: renal injury induced by I/R was associated with a significant reduction of urinary nitric oxide (NO) metabolites (NO₂/NO₃), and this effect was prevented by an MR blockade. B–D: effect of spironolactone administration on endothelial nitric oxide synthase (eNOS) expression and eNOS phosphorylation was evaluated by Western blot analysis using specific eNOS and phospho-eNOS antibodies. Renal eNOS expression and phosphorylation were not altered by I/R injury. In contrast, significant changes were observed in treated I/R groups. eNOS protein expression was increased by 1.5- to 2-fold in spironolactone-treated rats. In addition, the amount of phospho-eNOS S1177 was increased, while phospho-eNOS T497 was reduced. Error bars represent SE. *P < 0.05 vs. all compared groups. **P < 0.05 vs. sham and I/R groups.
DISCUSSION

In the present study, we show the novel finding that spironolactone administration protects the kidney against I/R injury. Specifically, our data show that pretreatment for 1, 2, or 3 days before rats were subjected to renal I/R prevented 1) renal dysfunction, 2) histological signs of tubular injury evidenced also by a reduction of urinary protein and NAG excretion, and 3) reperfusion injury supported by reduction of kidney lipoperoxidation and cell death by apoptosis.

The mechanisms of renal acute injury induced by I/R seem to be multifactorial and interdependent and involve hypoperfusion, hypoxia, inflammatory responses, and free radical-induced damage. The first step in initiating the pathophysiology of ischemic acute renal failure is renal blood flow reduction (27). In fact, we observed that renal blood flow and creatinine clearance remained significantly lower 24 h after renal I/R compared with sham-operated rats (Fig. 1). These alterations were accompanied by a significant increase in serum aldosterone levels (Fig. 3). There is increasing evidence to support potential roles of aldosterone in the pathogenesis of renal injury (3, 30). Here, we shown that administration of spironolactone before induction of renal I/R prevented renal blood flow and renal function reduction, suggesting that aldosterone promotes renal vasoconstriction and plays a potential role in the pathophysiology of acute renal failure. In support of these observations, previous studies from our laboratory showed that spironolactone prevented renal vasoconstriction induced by cyclosporine (8, 32). In addition, Arima et al. (1) demonstrated that aldosterone causes vasoconstriction in afferent and efferent rabbit arterioles, and, more recently, Gros et al. (12) reported that aldosterone mediated a dose-dependent contraction in clonal adult human vascular smooth muscle cells, which was inhibited by spironolactone and eplerenone, suggesting that the vasoconstrictor effect was due to the MR blockade. In this study, in addition of its profibrotic effects, we observed that aldosterone participates in promoting renal vasoconstriction during renal I/R, an effect that was prevented by spironolactone, implying that aldosterone induces renal vasoconstriction by a mechanism that requires the coupling of aldosterone to its receptor. In support of this possibility, a recent study shows that aldosterone induced vasoconstriction by decreasing the endothelial expression of glucose-6-phosphatase dehydrogenase, which, in turn, decreased the NO availability, and these effects were reversed by spironolactone administration, implying that the MR is involved (23). It has been reported that aldosterone exerts its actions by genomic and nongenomic mechanisms. The first is dependent on the classic MR, which promotes or prevents the transcription of certain genes, whereas the second seems to be mediated by an “unknown receptor,” which mediates fast actions independently of gene transcription (for a review, see Ref. 29). In this regard, it is known that the MR is a protein heterocomplex that includes a steroid-binding protein receptor and heat shock proteins (HSPs) of 56, 70, and 90 kDa. The presence of HSPs actually increases the receptor affinity for binding aldosterone, and when hormone binds to its receptor, HSPs are released. Interestingly, Tumlin et al. (45) reported that HSPs released are capable of activating calcineurin phosphatase. Thus it is possible that by its binding to the MR, aldosterone induces responses by at least two different mechanisms: by the classic pathway at the transcriptional level and by a nongenomic mechanism associated with the effects that occur through HSP release. Thus both genomic and nongenomic mechanisms could be dependent on the aldosterone binding to the classic MR.

As mentioned above, the decrease in renal blood flow is of critical importance in initiating and extending the pathophysiology of acute renal failure. Vasomotor tone is strongly affected by NO derived from eNOS. While NO derived from inducible NOS may contribute to the ischemic injury of renal tubules, there is evidence that the vascular effect of NO derived from eNOS in glomerular afferent arterioles is protective against I/R damage (41). Indeed, decreased eNOS function is one of the features of endothelial dysfunction associated with acute renal failure (11). In this regard, increased eNOS activity induced by ischemic preconditioning protected the kidneys from I/R (51). Also, the inhibition of Rho kinase in rats which underwent renal I/R preserved renal blood flow by improving eNOS function (46). These studies indicate an important role for eNOS activity as a protector against renal I/R. We observed, in the present study, a significant reduction in the amount of NO2/NO3 excreted in the urine after 24 h of renal I/R. Intriguingly, spironolactone prevented the reduction of these NO metabolites in the urine, suggesting that the improvement of NO generation is another mechanism associated with
protection induced by an MR blockade. It is possible that this effect of spironolactone is largely responsible for renal blood flow preservation in I/R spironolactone-treated rats. However, to analyze other possible mechanisms by which spironolactone improved NO production, the amount of eNOS and phosphorylation of active and inactive eNOS was determined. Phosphorylation of serine residue S1177 of eNOS is associated with activation of this enzyme, whereas phosphorylation of threonine residue T497 decreased its activity (42). When the spironolactone-pretreated I/R group was compared with the I/R untreated group (A and E), a significant reduction was seen in treated I/R groups, which was practically absent when spironolactone was given 2 or 3 days before I/R. I/R injury was associated with an increase in pro-caspase 3 mRNA kidney levels that was reduced by spironolactone pretreatment (K). Error bars represent SE. *P < 0.05 vs. sham-operated rats. **P < 0.05 vs. all groups.

It is well known that hypoxia, as a result of ischemia and subsequent reperfusion, is characterized by increased reactive oxygen species (ROS) and decreased efficacy of the antioxidant system, which lead to tubular cell injury and death (17). In the present, study we observed that renal I/R produced significant tubular damage at a histological level, as was evidenced also by the elevation of the amount of urinary protein and NAG excretion as tubular injury markers. Tubular damage observed was associated with an increase in renal TBARS contents as a marker of ROS generation (Fig. 5). Furthermore, a MR prophylactic blockade normalized renal TBARS levels and prevented the development of tubular injury. In support to these observations, it has been demonstrated that aldosterone in-
duced ROS generation by NADPH oxidase activation in cultured adult rat ventricular myocytes and mesangial cells (26, 39). Our results suggest that aldosterone may also contribute to induce ROS generation during a process of I/R. Thus, in addition to preventing renal hypoperfusion, MR antagonism reduced ROS generation and increased the mRNA levels of antioxidant enzymes (SOD and GPx), resulting in the preservation of tubular renal structure, as was shown by the light microscopic findings and the normalization of tubular injury markers.

Ischemic renal injury has been traditionally associated with tubular cell necrosis. However, apoptosis has emerged as a significant mode of cell death during renal I/R (20). Recent reports have demonstrated that interference with the apoptotic program translates into a protective effect during renal ischemia (6, 18), recognizing that the pathways associated with apoptosis may be very critical in the cell injury observed during I/R. In this study, untreated rats that underwent renal I/R presented a significant elevation of cell death by apoptosis in subcortical and juxtamedullary sections. The renoprotective effect of spironolactone was also associated with an important reduction of apoptosis in these sections. Because hypoxia and increased free radical generation resulting from renal hypoperfusion and reperfusion are known to trigger cell death by apoptosis, it is possible that the reduction of apoptosis observed in spironolactone-treated rats may result from the improvement of renal plasma flow (Fig. 1C) and decreased renal tissue lipoperoxidation (Fig. 5A).

In summary, in this study we show that aldosterone plays a central role in renal injury induced by I/R and emphasizes that spironolactone administration for 24–96 h before induction of renal I/R injury prevents the renal dysfunction and structural damage observed in this model. The mechanism of protection includes preservation of renal plasma flow and reestablishment of urinary NO2/NO3 excretion that was accompanied by increased expression of eNOS and phosphorylation at its residue S1177, and reduction of lipoperoxidation and cell apoptotic death. Based on our results, it will be intriguing to investigate the potential role of spironolactone in other models of renal injury, such as the cold ischemia associated with renal transplantation and I/R in other organs as that occurs in myocardial infarction treated with angioplasty. Our results may open new therapeutic avenues for the prevention of tissue damage in patients that are expected to be exposed to renal I/R, such as renal transplantation, high risk cardiovascular surgery, or I/R in other organs.

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