Recovery from ischemic acute kidney injury by spironolactone administration

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Abstract

Background. Prophylactic mineralocorticoid receptor (MR) antagonism with spironolactone (Sp) in rats completely prevents renal damage induced by ischemia. Because acute renal ischemia cannot typically be predicted, this study was designed to investigate whether Sp could prevent renal injury after an ischemic/reperfusion insult.

Methods. Six groups of male Wistar rats were studied: rats that received a sham abdominal operation (S); rats that underwent 20 min of ischemia and reperfusion for 24 h (I/R) and four groups of rats treated with Sp (20 mg/kg) 0, 3, 6 or 9 h after ischemia.

Results. As expected, I/R resulted in renal dysfunction characterized by a fall in renal blood flow and glomerular filtration rate and severe tubular injury which was confirmed by a significant increase in tubular damage biomarkers including kidney injury molecule-1, heat shock protein 72 and urinary protein excretion. The renal injury induced by I/R was in part due to Rho-kinase, endothelin and angiotensin II type 1 receptor upregulation. Interestingly, Sp administration at 0 and 3 h after ischemia completely reversed and prevented the damage induced by I/R. The protection induced by Sp given 6 h after ischemia was partial, but no protection was observed by administering Sp 9 h after ischemia.

Conclusion. Our results show that MR antagonism administered, either immediately or 3 h after I/R, effectively prevented ischemic acute renal injury, indicating that spironolactone is a promising agent for preventing acute kidney injury once an ischemic insult has occurred.

Keywords: AKI treatment; endothelin; renal dysfunction; Rho-kinase

Introduction

Acute kidney injury (AKI) is characterized by an abrupt and sustained decline in the glomerular filtration rate (GFR) that leads to a wide spectrum of acute alterations in kidney function and structure. One prominent cause of AKI is renal ischemia that occurs in patients with low blood pressure as a result of a complication of several common clinical situations, such as severe cardiac failure or arrhythmia, generalized sepsis or excessive loss of blood or fluid during surgery or as a consequence of trauma. Thus, ischemic and nephrotoxic injuries are the major causes of AKI in native and transplanted kidneys [1]. AKI occurs in ~5% of hospitalized patients and up to 40–60% of intensive care unit patients [2]. Despite technical improvements in dialysis and clinical care, the prevalence of AKI has risen significantly in the last 15 years due to aging population and the rising pandemics of obesity, diabetes and hypertension [3, 4]. In addition to high morbidity and mortality during the AKI episode, once resolved, AKI may also lead to the development of chronic kidney disease (CKD) or increases the transition rate from pre-existing CKD to end-stage renal disease [5–8]. In addition, even a small change in kidney function due to a minor AKI is associated with a higher long-term mortality rate [9].

In addition to its renal effects as a mineralocorticoid hormone, aldosterone plays a prominent role in the pathophysiology of renal diseases [10–19]. Recent evidence suggests that aldosterone is a potent renal vasoconstrictor [20–22]. Supporting that, aldosterone modulates the tone of the renal vasculature, we have shown that a mineralocorticoid receptor (MR) blockade with spironolactone not only reduces the structural renal damage associated with cyclosporine (CsA) but also prevents renal dysfunction due to afferent and efferent vasoconstriction [23–25]. We have also shown that prophylactic treatment with spironolactone completely prevents renal dysfunction and histological signs of tubular injury from ischemia–reperfusion (I/R) injuries [26]. To determine whether the protective effect of spironolactone during ischemia is due to blocking MR or to an unknown effect of this drug, we demonstrated that adrenalectomy had similar effects to those observed with spironolactone treatment [27]. Altogether, these results support the hypothesis that spironolactone prevents AKI after I/R by blocking the MR and therefore suggest that aldosterone plays a central role in promoting renal damage induced by a renal ischemic insult.
At present, there are no specific treatments for AKI caused by ischemia or nephrotoxic agents for use in the common clinical practice. In hypotension, once ischemia has damaged the kidney, other than restoring renal perfusion pressure and avoiding nephrotoxicities, there is nothing that can currently be done to help prevent further damage or repair what has already occurred in the tubular cells. Therefore, prevention is a challenge clinicians face. In this regard, several treatments and experimental strategies have been advanced for the prevention of AKI, with different degrees of effectiveness, depending on the patient’s other risk factors and the strategy used. However, most of these treatments are only effective when they are prophylactically administered [28–33]. Unfortunately, the development of renal ischemia cannot be predicted. Because we previously observed that giving spironolactone several hours before an ischemic insult completely prevents the histological and biochemical consequences of I/R, we designed the present study to investigate whether MR antagonism could reverse the renal injury induced by ischemia/reperfusion once ischemia has already been established and to better understand the molecular mechanisms of the renoprotection involved.

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. Sixty-two male Wistar rats (270–315 g) were divided into six groups: sham-operated rats (S); animals subjected to bilateral renal ischemia for 20 min and 24 h of reperfusion (I/R) and four groups of rats that underwent bilateral renal ischemia for 20 min and reperfusion for 24 h, but also received one dose of spironolactone at 20 mg/kg by gastric gavage immediately after or at 3, 6 or 9 h after ischemia (Sp0, Sp3, Sp6 and Sp9, respectively). Two hours after ischemia, the animals were placed in metabolic cages at 22°C with a 12:12-h light–dark cycle and were allowed free access to water. All animals were studied 24 h of reperfusion. At least six animals per group were used for renal functional and biochemical studies, and the rest were used for molecular studies.

Renal ischemia/reperfusion (I/R) injury (surgical procedure)

The rats were anesthetized by intraperitoneal injections of sodium pentobarbital (30 mg/kg) and underwent abdominal incision and the dissection of bilateral renal pedicles. Renal ischemia was induced by using non-traumatic vascular clamps; placed on each pedicle for 20 min. After the clamps were released, the incision was sutured, and the animals were allowed to recover. Sham abdominal operated rats underwent anesthesia and renal pedicle dissection only.

Functional studies

After recovery, the animals were placed in metabolic cages with 12:12-h light–dark cycle and were allowed free access to water. Twenty-four hours after ischemia, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital and placed on a homeothermic table. The trachea, jugular veins, femoral arteries and bladder were catheterized and renal artery and renal pedicle dissection only.

Histological studies

At the end of the experiment, one kidney was removed and quickly frozen for molecular studies. The other kidney was perfused with phosphate buffer through the femoral catheter, preserving the mean arterial pressure. The kidney was fixed with 4% formaldehyde. After appropriate dehydration, kidney slices were embedded in paraffin, sectioned at 4μm and stained with routine periodic acid–Schiff and hematoxylin. Ten cortical and juxamedullary fields were recorded from each kidney slice using a digital camera mounted on a Nikon microscope (Nikon Instruments Inc., Japan). Digital microphotographs were recorded for each rat slide to assess the number of tubules with cast formation per field. We counted at least 400 tubules on each slide per animal. The damaged tubular area was expressed as the percentage of tubules with cast formation.

Oxidative stress determination

Renal lipoperoxidation. Malondialdehyde (MDA), a measure of lipid peroxidation, was assayed as previously reported [26]. Briefly, after homogenization of the tissue, the reaction was performed in a 15.4 mM solution of N,N-methyl-2-phenylindole in 15% of hydrochloric acid and heated at 45°C for 40 min. The mixtures were centrifuged at 3000 g for 15 min. The supernatant absorbance was read at 586 nm. MDA was quantified using an extinction coefficient of 1.1 × 10^4 M^-1 cm^-1 and expressed as nanomoles of MDA per milligram of protein. The tissue protein composition was estimated using the Lowry method.

Urinary hydrogen peroxide assay. The amount of hydrogen peroxide (H_2O_2) in urine was determined with an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Eugene, OR) according to the manufacturer’s instructions. The assay employed a standard curve of H_2O_2 (1–10 μM). A 50 μL of each urine standard was placed in a microplate; 50 μL of Amplex red reagent/HRP was then added, and the samples were incubated for 30 min at room temperature, protected from the light. In the presence of peroxidase, the Amplex reagent reacts with H_2O_2 to produce resorufin, a red-fluorescent oxidation product. Therefore, the plate was read at 560 nm. The H_2O_2 concentration in the samples was expressed as nanomoles per 24 h.

Molecular studies

RNA isolation and real-time reverse transcription–polymerase chain reaction. Total RNA was isolated from the kidneys using the TRizol method (Invitrogen, Carlsbad, CA) and checked for integrity using 1% agarose gel electrophoresis. To avoid DNA contamination, total RNA samples were treated with DNAase (DNAase I; Invitrogen). Reverse transcription was conducted with 1.0 μg of total RNA using 200 U of
the Moloney murine leukemia virus reverse transcriptase (Invitrogen). The messenger RNA (mRNA) levels of kidney injury molecule-1 (Kim-1), Rho-kinase, angiotensinogen and angiotensin II type 1 (AT1) receptor as well as prepro-endothelin and ETA and ETB endothelin receptors were quantified by real-time polymerase chain reaction on the ABI Prism 7300 Sequence Detection System (TaqMan; ABI, Foster City, CA). Primers and probes were ordered as kits: Rn00597703_m1 for Kim-1, Mm00485745_m1 for Rho-kinase, Rn00593114_m1 for angiotensinogen, Rn00578456_m1 for AT1 receptor, Rn00560677_s1 for AT2, Rn00561129_m1 for prepro-endothelin, for the ETA receptor and Rn00560139_m1 for the ETB receptor (Assays-on-Demand; ABI). As an endogenous control, we used eukaryotic 18S ribosomal RNA (prede- signed assay reagent, external run; ABI). The relative quantification of all gene expression was performed using the comparative 2-\(\Delta\Delta C(t)\) method [37].

**Western blot analysis**

To obtain a pool of cell homogenized kidneys cells, proteins from the cortex of four kidneys in each group were isolated by homogenization with a lysis buffer. The denatured proteins were separated with a sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to polyvinylidene fluoride membranes (Millipore). The membranes were blocked and then incubated overnight at 4°C with goat anti-Rho-kinase antibody (1:2500; Santa Cruz Biototechnology, Santa Cruz, CA), rabbit anti-endothelin A receptor antibody (1:5000; Abcam Inc. Cambridge, MA), rabbit anti-endothelin B receptor antibody (1:5000; Abcam Inc.) or goat anti-angiotensin AT1 receptor (1:500; Abcam Inc.). Then, the membranes were incubated with a secondary antibody donkey anti-goat IgG-HRP (Santa Cruz Biotechnology) or rat anti-rabbit IgG HRP (Alpha Diagnostics, San Antonio, TX). To control protein loading and transfer, all membranes were probed with an actin antibody (1:5000) and a secondary antibody, donkey anti-goat IgG-HRP (1:5000; Santa Cruz Biotechnology). For this purpose, the membranes were stripped and re-probed. For stripping, the membranes were submerged in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCL, pH 6.7) and incubated at 50°C for 30 min, then the membranes were washed three times with large volumes of wash buffer and were blocked and incubated with the antibodies as described previously. In the case of Rho-kinase, the lower part of the membrane was used for actin blotting. Proteins were detected with an enhanced chemiluminescence kit (Millipore) 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.

**Tissue levels of endothelin-1**

Endothelin-1 levels were analyzed using a commercially available ELISA kit [Endothelin-1 (1-31) Assay kit; Immuno-Biological Laboratories Inc.] according to the manufacturer’s instructions. Tissue homogenates and standards were added to the pre-coated wells and incubated overnight at 4°C. Endothelin-1 was captured by the antibody and then detected by adding the labeled antibody and the chromogen. The optical density of the samples was read at 450 nm by a plate reader and was compared to a standard curve generated from known concentrations of endothelin-1 that ranged from 1.56 to 200 pg/mL. The protein concentration in the tissue homogenates was determined by the Lowry method (BioRad). The endothelin-1 concentration was normalized by the amount of protein added to the well.

**Statistical analysis**

Results are presented as the means ± Standard error. The significance of the differences among groups was tested by analysis of variance (ANOVA) using Bonferroni’s correction for multiple comparisons. Statistical significance was defined as having a P-value <0.05.

**Results**

Figure 1 depicts results of several physiological parameters analyzed in the studied groups. Neither I/R or spironolactone administration modified the mean arterial pressure values among the groups (Figure 1A). As we previously reported [26, 27], renal ischemia induced a significant increase in the serum aldosterone. A similar increase was observed in all experimental groups receiving spironolactone after the renal I/R insult (Figure 1B). After 24 h of ischemia, rats exposed to I/R exhibited renal hypoperfusion, as demonstrated by a significant decrease in RBF, and hypofiltration, as shown by the reduction in the
GFR (Figure 1C and D, respectively) and creatinine clearance (from 1.65 ± 0.13 to 0.86 ± 0.13 mL/min, P < 0.05). RBF reduction was not observed in the groups that received a single dose of spironolactone at 0, 3, 6 or 9 h after renal ischemia. The fall in GFR was partially prevented by spironolactone administration at 0, 3 and 6 h after ischemia as is depicted by GFR mean values (Figure 1D) and creatinine clearances (1.28 ± 0.12, 1.36 ± 0.15 and 1.79 ± 0.44 mL/min, respectively, although only in Sp6 group, the difference was statistically different by ANOVA). Even though the renal plasma flow was corrected in the 9-h group, the renal function did not recover. The upper panel of Figure 2 shows representative images of renal histological slides from the rats that underwent I/R alone (Figure 2A cortex, Figure 2B medulla) and from rats that underwent I/R followed by spironolactone at 0, 3, 6 and 9 h after bilateral renal ischemia (Figure 2C–J, respectively). Light microscopy revealed that renal I/R produced severe tubular damage characterized by lumen dilatation or collapse, loss of the brush border, cellular detachment from tubular basement membranes observed in the renal cortex (Figure 2A, magnification ×400) and extensive cast formation in renal medulla (Figure 2B, magnification ×100). All these lesions were practically absent in rats exposed to spironolactone at 0, 3 and 6 h after ischemic insult (Figure 2C–H, respectively). Consistent with the functional data, at the histological level, no protection was observed when spironolactone was administrated 9 h after ischemia was induced (Figure 2I and J).

Quantitative analysis of the histological images revealed that the percentage of tubules with cast formation in the I/R group was 23.9 ± 5.7%. In contrast, due to spironolactone administration, this percentage was reduced to 2.5 ± 1.3, 6.2 ± 2.2, 11.4 ± 3.7 and 8.7 ± 3.7 in the groups Sp0, Sp3, Sp6 and Sp9, respectively (Figure 2K), but only the values for Sp0 were significantly different from the I/R group, when ANOVA analysis was performed.
The biomarkers of tubular injury were consistent with the functional and histological observations. In the I/R group, urinary protein excretion increased by ∼4-fold, when compared with sham-operated rats (Figure 2L). In the rats that underwent I/R and were treated with spironolactone after 0 h, the increase in urinary protein excretion was completely prevented. In the Sp3, Sp6 and Sp9 groups, proteinuria developed, but the levels were significantly lower than those observed in the I/R group; however, only the Sp3 group showed levels that were significantly different. As shown in Figure 2M, Kim-1 mRNA levels increased by ∼150-fold in the I/R group, whereas in the Sp0 and Sp3 groups, Kim-1 mRNA levels were similar to the normal values. Although Kim-1 mRNA levels were increased in the Sp6 group, these values were significantly lower than those of the I/R group. In contrast, in the Sp9 group, Kim-1 upregulation was not prevented. We also assessed the urinary concentration of the Hsp72 because recent observations from our laboratory suggest that this protein is a sensitive and early biomarker of AKI induced by renal ischemia [38]. Accordingly, urinary Hsp72 excretion was significantly increased in the I/R group compared to the sham-operated rats (7.3 ± 1.3 versus 0.1 ± 0.03 ng/mL, respectively, P < 0.0001). Spironolactone administrated immediately after bilateral renal ischemia induction prevented Hsp72 upregulation (1.1 ± 0.3 ng/mL), but this upregulation was partially prevented when the mineralocorticoid blocker was administrated 3 or 6 h after the insult (3.2 ± 0.6 and 5.5 ± 1.1 ng/mL, respectively). The decrease in urinary Hsp72 excretion was not observed in the Sp9 group (7.0 ± 0.5 ng/mL).

The renoprotection conferred by early spironolactone administration after renal ischemia was also supported by the measurement of kidney malondialdehyde and urinary H₂O₂ excretion as markers of oxidative stress (Figure 3A and B, respectively). As we previously reported [26, 27], renal injury induced by I/R was associated with an ∼5-fold increase in oxidative stress in both tissue and urine. This increase in oxidative stress was not observed in the groups that received spironolactone at 0 or 3 h after kidney ischemia and this effect was observed in lesser

**Fig. 3.** Effect of spironolactone administration after ischemic insult on oxidative stress induced by I/R. (A) Renal malondialdehyde levels in sham-operated rats (white bars), in rats that underwent 20 min of bilateral renal ischemia and 24 h of reperfusion (black bars) and in I/R rats that received spironolactone immediately, 3, 6 or 9 h after renal ischemia (gray bars, Sp0, Sp3, Sp6 and Sp9, respectively). (B) Urinary H₂O₂ excretion in all studied groups. *P < 0.05 versus sham-operated rats, ψP < 0.05 versus the I/R group.

**Fig. 4.** Ischemia/reperfusion injury is associated with Rho-kinase upregulation and is partially prevented by early spironolactone administration. (A) Rho-kinase mRNA levels determined by real-time reverse transcription–polymerase chain reaction in total renal cortex RNA extracted from sham-operated rats (white bars), in rats that underwent 20 min of bilateral renal ischemia and 24 h of reperfusion (black bars) and in I/R rats that received spironolactone immediately, 3, 6 or 9 h after renal ischemia (gray bars, Sp0, Sp3, Sp6 and Sp9, respectively). (B) The upper inset is a representative autoradiography image obtained from a western blot analysis of all studied groups. The graph represents the densitometry analysis. Error bars represent the Standard error.
proportion in the rats treated after 6 h. This renoprotective effect was not observed in the Sp9 group.

Recent studies have shown that Rho-kinase is involved in signaling pathways that mediate vasoconstriction [39]. Therefore, its induction by aldosterone might contribute to the renal hypoperfusion and hypoxia observed after an ischemic insult. To determine whether these effects are another potential renoprotective mechanism of spironolactone, the expression levels of Rho-kinase were assessed. Figure 4A shows the mRNA levels of Rho-kinase and Figure 4B the Rho-kinase protein expression. In the kidneys from rats with ischemia and reperfusion, the mRNA and protein levels of Rho-kinase were increased compared to those of the sham-operated rats; however, the differences did not reach significance by ANOVA analysis. Of note, this upregulation was partially prevented in the groups treated with spironolactone immediately and 3 h after renal ischemia, but not in the groups that received spironolactone 6 or 9 h afterward.

Endothelin is a potent vasoconstrictor in renal vasculature; thus, we also assessed the mRNA levels of prepro-endothelin, the tissue endothelin levels by ELISA and the levels of ETA and ETB receptors by western blot analysis. In accordance with our previous observations [27], prepro-endothelin mRNA levels increased by ∼9-fold in the I/R group, as is shown in Figure 5A. The increase in prepro-endothelin transcripts was partially prevented by spironolactone administration, even after 9 h. An increase in endothelin after renal ischemia was also observed by using ELISA and was prevented by the administration of spironolactone immediately after ischemia, the differences between I/R rats and those treated at 3, 6 or 9 h were not significant by ANOVA (Figure 5B). This is probably due to the similarity between the I/R group and most of the groups treated with spironolactone; however, it is evident that endothelin was increased by ischemia, indeed t-test was significant when this group was compared with sham-operated values, and that this increase was prevented by spironolactone administered at time 0. Renal ischemia had no effect upon the expression of the ETA receptor (Figure 5C). In contrast, the level of the vasodilatory ETB receptor was significantly reduced in the I/R group (Figure 5D). This effect was partially prevented by spironolactone at 0, 3 and 6 h, but not at 9 h.

Angiotensinogen and the AT1 receptor mRNA levels were measured in the total RNA extracted from the kidneys in each study group. No changes in angiotensinogen mRNA levels were observed (Figure 6A), but the I/R

Fig. 5. Ischemia/reperfusion injury is associated with endothelin upregulation and is partially prevented by spironolactone administration. (A) Prepro-endothelin mRNA levels determined by real-time reverse transcription–polymerase chain reaction in total renal cortex RNA extracted from sham-operated rats (white bars), in rats underwent 20 min of bilateral renal ischemia and 24 h of reperfusion (black bars) and rats that received spironolactone immediately, 3, 6 or 9 h after renal ischemia (gray bars, Sp0, Sp3, Sp6 and Sp9, respectively). (B) Renal cortex endothelin levels assessed by ELISA in all studied groups. (C) and (D) ETA and ETB protein levels by western blot analysis; the upper insets are representative images of this chemiluminescent analysis and the lower graphs are the corresponding densitometric analysis. *P < 0.05 versus sham-operated rats, ψP < 0.05 versus the I/R group.
group exhibited more than a 10-fold upregulation of AT1 mRNA, and this effect was completely mitigated in all of the groups treated with spironolactone after the ischemic insult, as depicted in Figure 6B. In accordance with these findings, western blot analysis depicted in Figure 6C, showed that AT1 receptor protein level was significantly increased in the I/R group by 3-fold. In contrast, AT1 receptor upregulation was significantly reduced in all the groups treated with spironolactone.

Discussion

In this study, we show that blocking the MR with spironolactone immediately after or even 3 h after renal bilateral ischemia completely prevented renal dysfunction, tubular injury and oxidative stress. The expected increase in sensitive biomarkers of renal injury was also prevented. A direct relationship between renal protection and the blockade of MR is also supported by the fact that spironolactone conferred partial renoprotection when administered after 6 h and did not confer protection after 9 h.

AKI is characterized by vascular and tubular abnormalities that take place after ischemia and during the reperfusion process. Intra-renal vasoconstriction that leads to a reduction in GFR, together with vascular congestion in the outer medulla and activation of tubulo-glomerular feedback constitute part of the vascular defects, which are likely caused by the increased release of vasoconstrictor factors (mainly endothelin, adenosine and angiotensin II) and decreased production of vasodilators (such as nitric oxide, prostaglandin, acetylcholine and bradykinin) (for a review [40]). Considering that hypoperfusion is a major stimulus of the renin–angiotensin–aldosterone system and that we previously showed that both prophylactic spironolactone administration and adrenals removal prevented renal dysfunction induced by I/R [26, 27], our data suggest that aldosterone plays a primary role in sustaining renal vasoconstriction in this model of ischemic damage. In our previous study using I/R as a model of AKI, the effects of aldosterone were blocked before the ischemic insult took place, and the resulting prevention of AKI was so remarkable that we reasoned that it is possible that spironolactone given after renal ischemia could be helpful to reduce or prevent functional and structural injury. Considering that renal ischemia often occurs unexpectedly in the clinical setting, we evaluated the ability of spironolactone to protect the kidney after establishing an ischemic insult. We found that the decline in RBF with the subsequent reduction in GFR induced by I/R was completely prevented when spironolactone was administrated immediately or 3 h after the renal ischemic insult had occurred. Administering spironolactone 6 h after the ischemic insult produced a lower degree of protection, which, however, was still significant. In this study, we used a low calorie commercial sugar as a GFR marker since we previously showed that this compound has enough sensitivity to measure GFR in normal and pathophysiological conditions to a similar extent of the gold standard polyfructosan [34, 41]. Moreover, our data were further confirmed when the renal function was assessed by creatinine clearance.

It is well known that the tubular epithelium suffers functional and morphological alterations as a consequence of renal hypoperfusion induced by ischemia. Hence, the I/R group exhibited the classical picture of acute tubular necrosis that was accompanied by increased urinary protein excretion, together with upregulation of sensitive biomarkers such as Kim-1 and Hsp72 [38, 42-44]. The morphological alterations and the elevation of sensitive tubular markers of renal injury were also prevented or reduced when spironolactone was administrated immediately and until 6 h after renal ischemia had occurred. Hsp72 was the most consistent biomarker with the time-dependence protection of spironolactone evidenced by the histological injury. These discrepancies among the biomarkers used could be due to a greater sensitivity of Hsp72 to stratify renal injury than Kim-1 or proteinuria, as we previously reported [38] or with different kinetics of induction of each biomarker because it has been
reported that some biomarkers are induced very quickly and returned to normal values after 24 h of renal injury or contrariwise [45]. Taken together, our observations provide evidence about the potential effect that the mineralocorticoid blockade may have on the improvement of renal function and structure when spironolactone is administered immediately or even 6 h after an ischemic insult is established. This action spectrum might be more notable in humans, considering the short life of the rats.

As expected [46], the extensive tubular damage observed in the I/R group was associated with a significant increase in oxidative stress, as was evidenced by the increase in renal thiobarbituric acid reactive substances and in the urinary excretion of $\text{H}_2\text{O}_2$. This was also prevented by the MR blockade 0, 3 or even 6 h after renal ischemia, suggesting that aldosterone promotes a cellular oxidative milieu. In support of these findings, a specific role for aldosterone in mediating oxidative stress has become apparent [47], specifically; it has been shown that aldosterone induced reactive oxygen species generation by inducing the activation of NADPH oxidase in cultured mesangial cells [48] and by decreasing glucose-6-phosphate dehydrogenase activity [22].

Recent advances in vascular cell biology have demonstrated 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 (for a review, see [39]). Specifically for AKI, during renal I/R, Rho-kinase mRNA and protein levels increase [27, 49]. Renal injury induced by long-term aldosterone administration has also been associated with increases in myosin phosphate target subunit-1, a marker of Rho-kinase activity [50]. Thus, the Rho/Rho-kinase pathway has recently attracted great attention in various research fields, due to its participation in vascular tone regulation [51] and more recently, due to its role in mediating both extensive foot-process effacement and histologic features of focal segmental glomerulosclerosis [52]. In the present study, we detected that in kidneys isolated from I/R rats that exhibited considerable increase in renal toxification of NADPH oxidase in cultured mesangial cells [48] and by decreasing glucose-6-phosphate dehydrogenase activity [22].

As was previously mentioned, the reduction in RBF is primarily mediated by an imbalance in vasoactive substance release. Endothelin is a largely vasoconstrictive peptide that has been implicated in renal pathophysiology [55]. An increase in endothelin levels during I/R-induced renal injury has been observed by us as well as by others [27, 56]. Here, we corroborated that I/R-induced renal injury was related with an upregulation of prepro-endothelin mRNA levels and renal endothelin level. These transcriptional changes were prevented in the animals that underwent ischemia and received spironolactone at 0, 3 and 6 h, suggesting that aldosterone mediates this transcriptional effect. In fact, several studies have demonstrated an increase in both endothelin-1 transcript and protein in response to aldosterone in vascular smooth muscle [57], cardiac [58] and renal [56] tissues as well as in inner medullary collecting duct cells [59]. Interestingly, responsive elements to MRs have been identified in the endothelin promoter [60]. Moreover, endothelin upregulation may also increase Rho-kinase activity [58], prolonging the vicious cycle that contributes to the adverse effects of aldosterone under pathophysiological conditions.

As mentioned above, angiotensin II is a mediator of renal injury induced by I/R. We previously showed that blocking AT1 receptor with losartan partially prevents renal dysfunction in rats that have undergone ischemia [61], suggesting that this peptide partially participates in the renal vasoconstriction observed in this model of renal damage. Although we did not observe changes in the angiotensionogen mRNA levels, the AT1 receptors were upregulated at the mRNA and protein levels in rats that underwent ischemia/reperfusion, an effect that was completely prevented by spironolactone.

We recently observed that the prophylactic treatment with MR blocker spironolactone completely prevented I/R-induced AKI in rats [26]. Here, we showed a similar degree of protection when spironolactone was administered after the ischemic insult had been established. The renoprotection conferred by spironolactone was mediated by slowing down endothelin, AT1 receptor and Rho-kinase renal levels that contributed to preventing renal hypoperfusion and the concomitant generation of free radicals. These data together with our previous findings show that MR antagonism should be further studied as a strategy for preventing AKI following renal ischemia.

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Conflict of interest statement. None declared.
References

15. K. Sánchez-Pozo et al.
ARB protects podocytes from HIV-1 nephropathy independently of podocyte AT1

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Abstract

Background. Angiotensin I-converting enzyme inhibitors and angiotensin receptor blockers protect podocytes more effectively than other anti-hypertensive drugs. Transgenic rats overexpressing angiotensin II Type 1 (AT1) receptor selectively in podocytes have been shown to develop glomerulosclerosis. The prevailing hypothesis is that angiotensin II has a capacity of directly acting on the AT1 receptor of podocytes to induce injury. We therefore investigated the mechanism of reno-protective effect of AT1 receptor in a mouse model of HIV-1 nephropathy.

Methods. We generated transgenic mice carrying the HIV-1 gene (control/HIV-1) or both HIV-1 gene and podocyte-selectively nullified AT1 gene (AT1KO/HIV-1). In these mice, we measured urinary protein or albumin excretion and performed histological analysis.

Results. At 8 months of age, AT1KO/HIV-1 (n = 13) and control/HIV-1 (n = 15) mice were statistically indistinguishable with respect to urinary albumin/creatinine ratio (median 2.5 versus 9.1 mg/mg), glomerulosclerosis (median 0.63 versus 0.45 on 0–4 scale) and downregulation of nephrin (median 6.90 versus 7.02 on 0–8 scale). In contrast to the observed lack of effect of podocyte-specific AT1KO, systemic AT1 inhibition with AT1 blocker (ARB) significantly attenuated proteinuria and glomerulosclerosis in HIV-1 mice.

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