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

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Abstract

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 (8; 32). 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 underwent 20 min of ischemia and 24 h of reperfusion, and three groups 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 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 NO_2/NO_3 excretion. Endothelial nitric oxide synthase expression was up-regulated by a MR 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 was observed. In conclusion, our study shows that spironolactone administration prevents the renal injury induced by ischemia/reperfussion, suggesting that aldosterone plays a central role in this model of renal injury.

Introduction

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 a mineralocorticoid receptor (MR) blockade improves patients survival with chronic heart disease (29; 30) (33; 34) and chronic renal failure (4; 5). The protective effect of a MR blockade is associated with decreased fibrosis and vascular inflammation, suggesting that aldosterone is a pro-fibrotic hormone (16; 19; 19). (19) In addition, effectiveness of MR antagonism in ameliorating glomerular and/or tubulointerstitial 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, a MR blockade reduced structural injury (32) and helps to avoid the progression of renal damage in a model of preexisting chronic CsA nephrotoxicity (31), by mechanisms that involved the reduction of TGFβ expression, lipoperoxidation, and cell death by apoptosis. Interestingly, in the course of these studies, we noticed that a MR blockade prevents the well known effect of CsA-inducing renal vasoconstriction (2; 7; 8). CsA administration in rats during 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 R, et al. (12) have recently shown 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-mediating 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 ischemia/reperfusion injury.

Material 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/k 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/k). 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 non-traumatic vascular clamps over the pedicles for 20 minutes. After clamps were released, the incision was closed in 2 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-hour urine samples were collected. Urinary protein excretion was measured by a TCA turbidimetric method (14) and N-acetyl-beta glucosaminidase (NAG) was measured spectrophotometrically (49). Serum and urine creatinine concentrations were measured with an autoanalyzer (Technicon RA-1000, Bayer Co. Tarrytown, NY) and renal creatinine clearance was calculated by the standard formula C= (U xV)/ P, where U is the concentration in urine, V is the urine flow rate, and P is

the serum concentration. Serum aldosterone was quantitatively determined by radiommunoassay following the procedures described by the manufacturer (DiaSorin S.p.A. 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 euvolemic conditions by infusing 10 ml/kg of body weight of isotonic rat plasma during surgery. The mean arterial pressure was monitored with a pressure transducer (Model p23 db, Gould. Puerto Rico) 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 (RBF).

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, 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 4μ, and stained via a 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. Affected tubular area was

analyzed blindly by an expert nephropathologist. Tubular damage was characterized by a loss of brush border, lumen dilatation or collapse, and detachment from 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 damaged tubular area, delimited by using eclipse net software (magnification, X400). The damaged tubular area was expressed as a proportion of the affected tubular area and total tubular area.

TUNEL assay. Apoptosis in kidney sections was determined by TUNEL assay using ApopTag *in situ* apoptosis detection kit (S7101, Chemicon International, Temecula, CA, USA). Slides were prepared by following the procedures previously described (50). A minimum of 10 subcortical and 10 juxtamedullary fields (magnification 400x) 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 lipoperoxidation. Malondialdehyde (MDA), a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reacting substance (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 3000 g for 15 minutes. 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 nmol of TBARS per milligram of protein. The tissue protein was estimated using the Bradford method.

RNA isolation and real time PCR. The total RNA was isolated from each kidney following the guanidine isothyocianate-cesium chloride method (40) and checked for integrity by 1% agarose gel electrophoresis. Reverse transcription (RT) 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 on the ABI Prism 7300 Sequence Detection System (*Taq*Man, Applied Biosystems ABI, 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 (pre-designed assay reagent Appplied by ABI, external run) 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 eNOS antibody, phospho eNOS T495 antibody or phospho eNOS S1177 antibody, all used at 1:500 (Cell Signaling Technology Inc). Afterward, membranes were incubated with a secondary antibody HRP-conjugated rat anti-rabbit IgG, 1:2500 (Alpha Diagnostics, San Antonio, TX). To control protein

loading and transfer, all membranes were simultaneously probed with an actin antibody 1:2500 and secondary antibody donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Santa Barbara, CA). Proteins were detected with an enhanced chemiluminescence kit (Amersham) and autoradiography, following 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 a mean ± SE. Significance of the differences among groups were tested by ANOVA using Bonferroni's correction for multiple comparisons. All comparisons passed the normality test. Statistical significance was defined when p value was <0.05.

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 hours 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 (Figures 1A and 1B). Renal impairment was, in part, related to renal plasma flow reduction by 33%, without changes in mean arterial pressure (Figures 1C and 1D). In contrast, in the three groups that were pre-treated with MR blocker spironolactone for one, two, or three days before renal I/R (sp1, sp2 and sp3 groups), the fall in renal plasma flow was completely prevented (Figure 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) (Figure 1A) and the concomitant reestablishment of renal function, as shown by the values of the creatinine clearance (Figure 1B).

Light microscopy 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 (Figure 2A and 2E; 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 (Figures 2B-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 I/R group was 66.2 ± 0.7 % compared to 6.1 ± 1.4, 9.1 ± 2.5 and 6.6 ±

2.0 in sp1, sp2 and sp3 groups, respectively (Figure 2I). An assessment of classical markers of tubular injury supported these observations. Proteinuria and N-acetyl-beta glucosaminidase (NAG) excretion increased 5-fold and 3-fold, respectively, in I/R group. Accordingly, in rats pre-treated with spironolactone, the levels of these tubular injury markers were similar to those found in sham-operated rats and significantly different from untreated I/R group (Figures 2J-K). Figure 3A shows that animals subjected to renal I/R presented a significant elevation of serum aldosterone levels by more than 5-fold compared to sham operated rats. Aldosterone levels were not different among I/R groups. As shown in Figure 3B, a 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 NO_2/NO_3 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 NO_2/NO_3 excretion, as shown Figure 4A. Thus, we analyzed the expression levels and phosphorylation state of endothelial nitric oxide synthase (eNOS). The eNOS expression levels and phosphorylation in S1177 and T497 residues were not affected by I/R, as shown in Figure 4B-D. Kidney eNOS protein levels were significantly increased in rats that received the prophylactic treatment with spironolactone (Figure 4B). In addition, spironolactone treatment was associated with an increase of the activating eNOS S1177 phosphorylation (Figure 4C) and a decrease of the inactivating eNOS T497 phosphorylation (Figure 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 3-fold, which was prevented by the prophylactic treatment with a MR blocker, since the first day of pre-treatment (Figure 5A). The reduction of lipoperoxidation observed in the spironolactone-treated groups was accompanied by a significant increase of superoxide dismutase and glutathione peroxidase as antioxidant enzymes, while the catalase mRNA levels did not change (Figure 5B-D).

In situ labeling of cell nuclei by a TUNEL method showed that I/R produced a significant increase in apoptosis cell death measured by positive nuclei stain per mm^2 that was more evident in juxtamedullary areas than in subcortical sections, as shown in Figures 6A and 6B. These observations were confirmed by the quantification of positive nuclei per mm^2, graphically expressed in Figure 6I, showing more than 100 and 300 positive nuclei/ mm^2 in subcortical and juxtamedullary kidney sections of rats that were subjected to renal I/R. Spironolactone pretreatment for one day before renal I/R reduced the number of positive nuclei in both areas (Figure 6B-C and 6I-J). Nevertheless, apoptosis was completely prevented when a MR blockade started two or three days before I/R (Figure 6C-6J). 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 (Figure 6K).

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 pre-treatment 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, as compared to 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). (30) Here, we shown that administration of spironolactone prior to inducing renal I/R prevented the RBF 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, that was inhibited by spironolactone and eplerenone, suggesting that vasoconstrictor effect was due to the blockade of mineralocorticoid receptor. In this study, in addition of its pro-fibrotic effects, we observed that aldosterone participates in promoting renal vasoconstriction during renal ischemia/reperfusion, an effect that was prevented by spironolactone, implying that aldosterone induces renal vasoconstriction by a mechanism that require the coupling of aldosterone to its receptor. Supporting this possibility, a recent study shows that aldosterone induced vasoconstriction by decreasing the endothelial expression of glucose-6-phosphate deshydrogenase, which in turns decreased the NO availability, and these effects were reverted by spironolactone administration, implying that mineralocorticoid receptor is involved (23). It has been reported that aldosterone exerts its actions by genomic and non-genomic mechanisms. The first is dependent on the classical mineralocorticoid receptor that promotes or prevents the transcription of certain genes, whereas, the second seems to be mediated by a “unknown receptor” that mediates fast actions independent of gene transcription (for review (29)). In this regard, it is known that mineralocorticoid receptor is a protein heterocomplex that includes steroid binding protein receptor and heat shock proteins (HSPs) of 56, 70 and 90KDa. The presence of HSPs actually incrase 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 to activate calcineurin phosphatase. Thus, it is possible that by its binding to mineralocorticoid receptor, aldosterone induces response by at least two different mechanisms: by the classical pathway at transcriptional level and by a non

genomic mechanism associated with the effects that occur by HSPs release. Thus, both genomic and non-genomic mechanism could be dependent on the aldosterone binding to the classical mineralocorticoid receptor.

As we 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 nitric oxide derived from endothelial nitric oxide synthase (eNOS). While nitric oxide derived from iNOS may contribute to the ischemic injury of renal tubules, there is evidence that the vascular effect of nitric oxide 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 NO_2/NO_3 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 the NO generation is another mechanism associated with protection induced by a MR blockade. It is possible that this effect of spironolactone is largely responsible of renal blood flow preservation in I/R spironolactone-treated rats. However, in order 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 pretreated spironolactone I/R group was compared with I/R untreated group, up-regulation of eNOS protein levels, increased amount in the phosphorylation of residue S1177 of eNOS, and reduced phosphorylation of the residue T497 of eNOS were observed. In support of these findings, it has been reported that aldosterone reduces NO generation in human umbilical vein endothelial cells by a mechanism that includes the reduction of eNOS S1177 phosphorylation and increased free radical production (28). Thus, increased amounts of activated eNOS in the kidney could be responsible for the observed increase in urinary NO_2/NO_3 excretion and reestablishment of renal blood flow in I/R spironolactone-treated animals.

It is well know that the hypoxia, as a result from ischemia and the subsequent reperfusion, is characterized by an increased reactive oxygen species (ROS) and decreased efficacy of antioxidant system, that 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 thiobarbituric acid reactive substances (TBARS) contents as a marker of ROS generation (Fig 4). 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 induced 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 ischemia/reperfusion. 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 microscopy 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 ischemia/reperfusion (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 ischemia reperfusion. 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 associated also with an important reduction of apoptosis in these sections. Because hypoxia and increased free radicals generation resulting from renal hipoperfusion 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 (Figure 1C) and decreased renal tissue lipoperoxidation (Figure 4A).

In summary, in this study we show that aldosterone plays a central role in renal injury induced by ischemia/reperfusion and emphasizes that spironolactone administration for 24 to 96 hours before induction of renal ischemia/reperfusion 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 NO_2/NO_3 excretion that was accompanied with 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 ischemia, such as the cold ischemia associated with renal transplantation and ischemia/reperfusion 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 ischemia reperfusion in other organs.

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Figure Legends

Figure 1. Renal functional parameters in sham, untreated I/R (I/R) and spironolactone-treated groups for one (Sp1), two (Sp2), and three (Sp3) days before renal I/R induction, as stated. I/R-induced renal failure as was evidenced by a significant increase of serum creatinine (A), together with a reduction of renal creatinine clearance (B) and renal blood flow, RBF (C), without changes in mean arterial pressure, MAP D) in the I/R untreated group. Renal dysfunction was completely prevented by I/R spironolactone-pretreated rats.

Figure 2. A-H Subcortical histological sections of kidneys stained with PAS from groups studied as stated. A-D lower power (100x) and E-H high power (400x) microphotographs. A and E representative microphotographs of a kidney section from an I/R untreated rat. Arrow heads 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 spironolactone-pretreated rats from one to three days (B-H). I) Morphometric quantification of affected tubular area, J) Urinary protein excretion levels and K) urinary N-acetyl-beta-glucosaminidase (NAG) excretion. Error bars represent s.e.m. **$p<0.05$ vs. all studied groups.

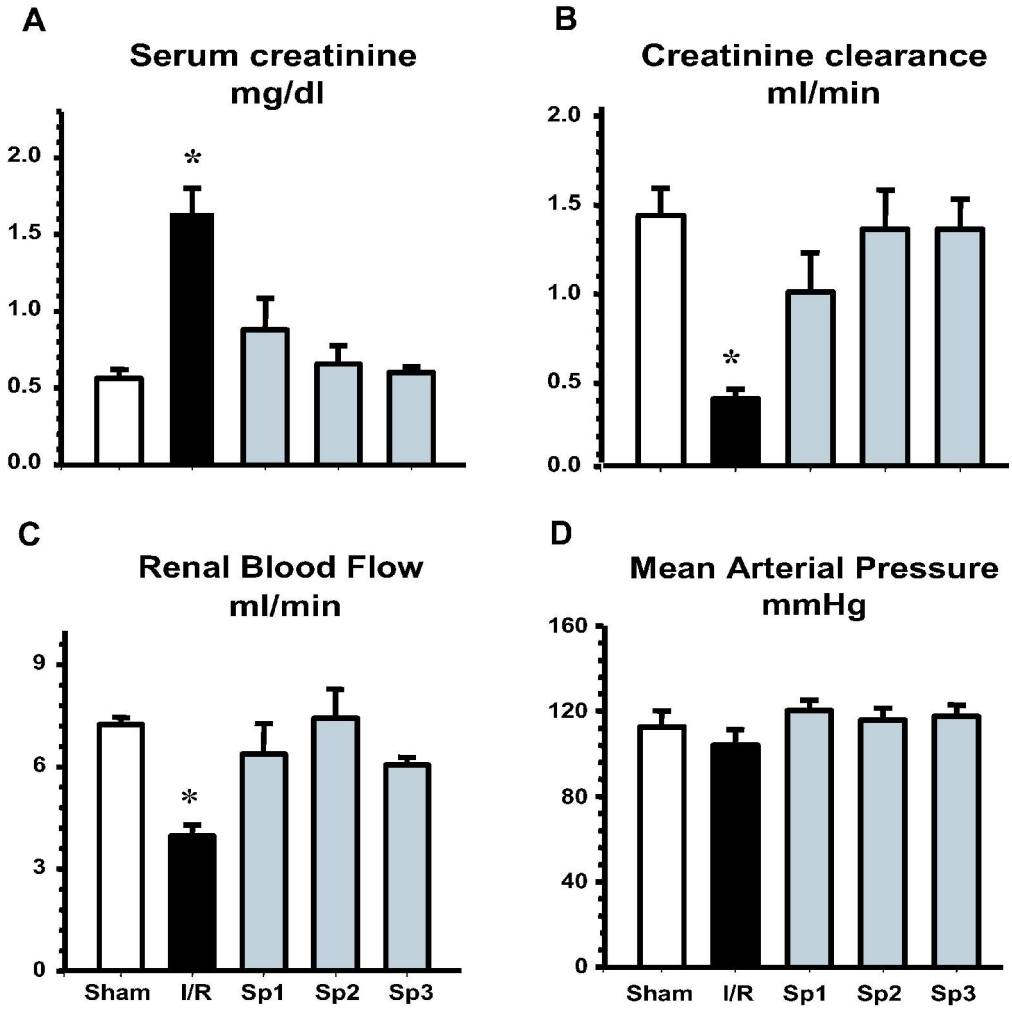
Figure 3. Effect of I/R and MR blockade treatment on serum aldosterone and potassium levels. A) All rats that were subjected to I/R presented a marked elevation of serum aldosterone levels compared to the sham-operated group. B) Neither renal I/R nor spironolactone treatment modified serum potassium levels. Error bars represent s.e.m. * $p<0.05$ vs. sham operated rats.

Figure 4. Renoprotective mechanisms of MR blockade in renal injury induced by I/R. A) The renal injury induced by I/R was associated with a significant reduction of urinary NO metabolites (UNO_2/NO_3V) and this effect was prevented by a MR blockade. B-D) The effect of spironolactone administration on eNOS and eNOS phosphorylation were evaluated by Western blot analysis using specific eNOS and phospho eNOS antibodies. Renal eNOS expression and phosphorylation was not altered by I/R injury. In contrast, significant changes were observed in treated IR 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 s.e.m. * $p<0.05$ vs. all compared groups. ** $p<0.05$ vs. sham and I/R groups.

Figure 5. Effect of renal I/R and MR blockade on renal lipoperoxidation and antioxidant enzymes mRNA levels. A) Spironolactone administration prevented the kidney lipoperoxidation induced by I/R (malondialdehyde MDA). This renoprotective effect was associated with increased mRNA levels of superoxide dysmutase and glutathione peroxidase (B-C), with no changes in catalase (D).

Figure 6. Apoptotic cellular death. (E-H). Representative subcortical and juxtamedullary kidney sections showing positive nuclei for apoptosis determined by terminal deoxynucleotide transferase-mediate dUTP nick end labeling (TUNEL) assay (coffee brown) in the studied groups, as indicated. Quantification of TUNEL-positive nuclei by mm^2 in subcortical and juxtamedullary areas (I-J). Note the marked increase in the number of TUNEL-positive cells (arrows) in I/R untreated group (A, 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 of pro-caspase 3 mRNA kidney levels that was reduced by spironolactone pretreatment (K). Error bars represent s.e.m. * $p<0.05$ vs. sham operated rats and ** $p<0.05$ vs. all the groups.

Figure 1



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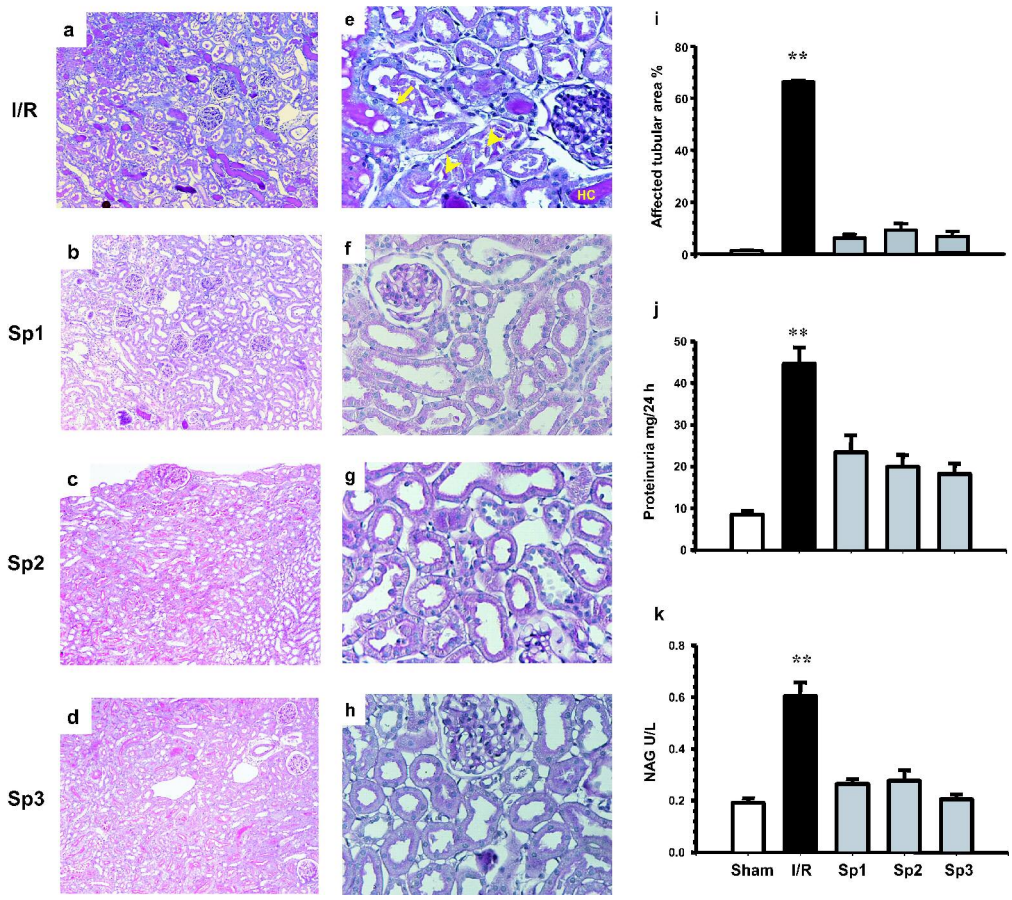

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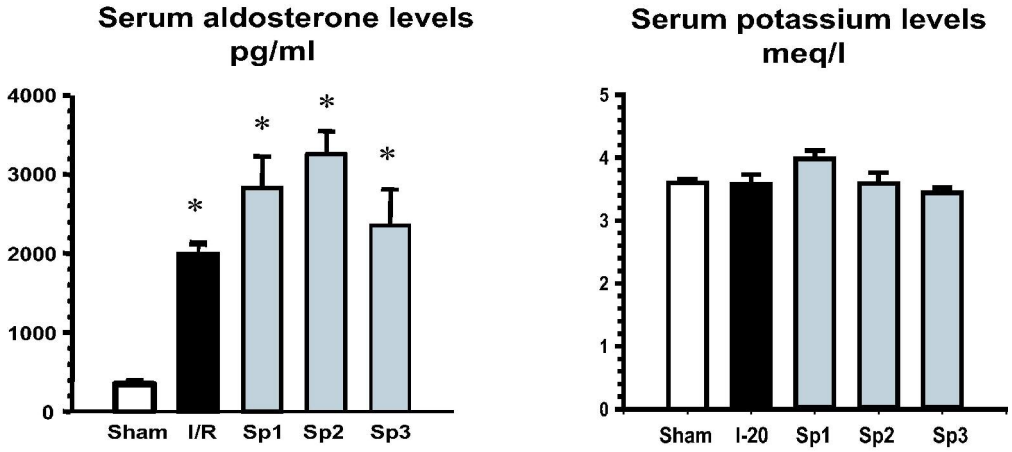

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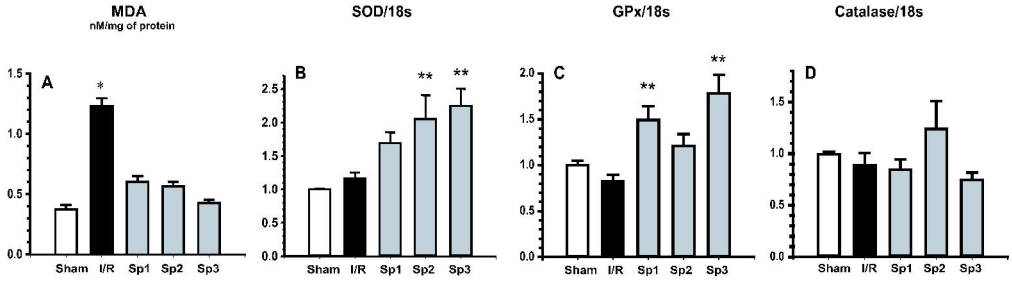

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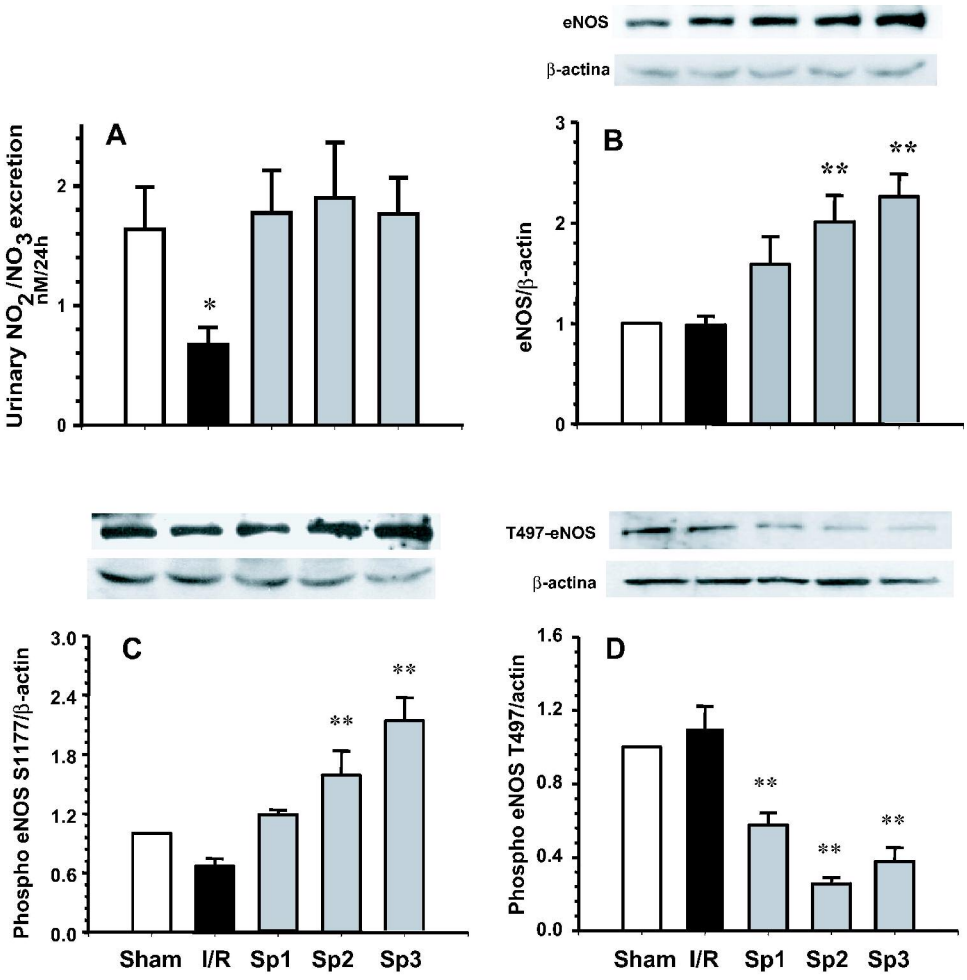

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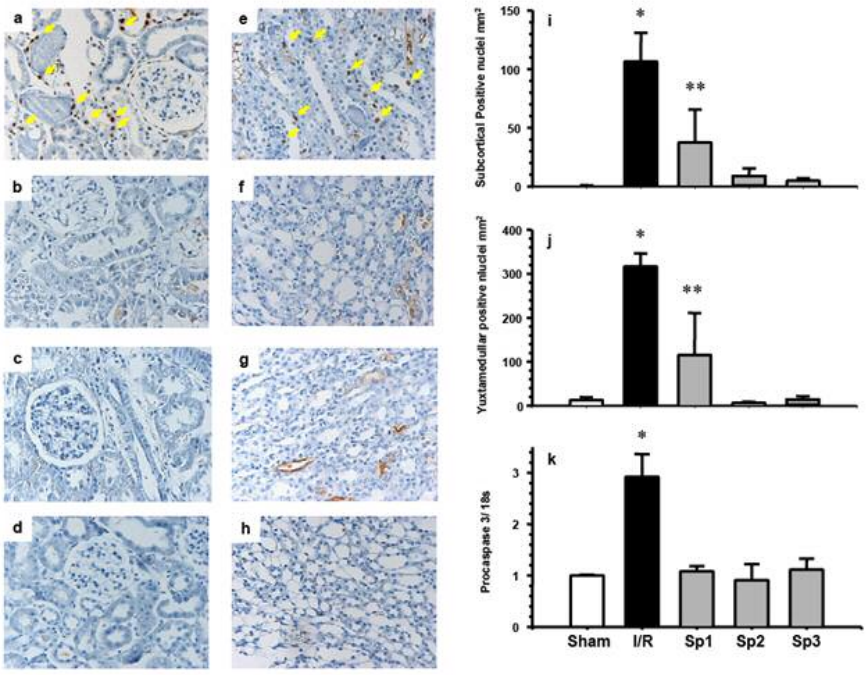

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