INSTITUTO NACIONAL DE
CIENCIAS MÉDICAS
Y NUTRICIÓN
SALVADOR ZUBIRÁN

México, Cd.Mx., a 8 de Mayo del 2018

Dr. Jorge Barrios Payan
Secretario de la Comisión de
Investigación en Animales CINVA
P R E S E N T E .

Estimado Dr. Barrios:

Anexo a la presente le hago llegar el informe técnico final del protocolo: "**Implicación de la Aldosterona en el desarrollo de enfermedad renal crónica como consecuencia de una lesión renal aguda**" con registro **CINVA: NMN-45-11-13-1.** Adjunto encontrará los comprobantes que lo avalán.

Sin otro particular por el momento, quedo de usted.

Atentamente,

Dra. Norma A. Bobadilla Sandoval
Investigador en Ciencias Médicas F
Depto. Nefrología y Metabolismo Mineral

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Informe Técnico final del proyecto

"Implicación de la Aldosterona en el desarrollo de enfermedad renal crónica como consecuencia de una lesión renal aguda"

Responsable: Dra. Norma A. Bobadilla Sandoval

A continuación, se destacan los productos obtenidos durante la ejecución y finalización del proyecto con registro **CINVA: NMN-45-11-13-1.**

Publicaciones en revistas internacionales indizadas:

1) Rodríguez-Romo R, Benítez K, Barrera-Chimal J, Pérez-Villalva R, Gómez A, Aguilar-León D, Rangel-Santiago JF, Huerta S, Gamba G, Uribe N, and Bobadilla NA. Autor Corresponsal: Bobadilla NA. AT1 receptor antagonism before ischemia prevents the transition of acute kidney injury to chronic kidney disease. Kidney Int Feb 89(2) 363-373, 2016. FI: 8.6

2) Barrera-Chimal J, Bobadilla NA, Jaisser F. Mineralocorticoid Receptor Antagonism: A Promising Therapeutic Approach to Treat Ischemic AKI. Nephron. 134(1):10-3. 2016. FI: 1.6

3) Barrera-Chimal J, Pérez-Villalva R, Ortega JA, Sánchez A, Rodríguez-Romo R, Durand M, Jaisser F, and Bobadilla NA. Autor Corresponsal: Bobadilla NA. Mild ischemic injury leads to long-term alterations in the kidney: amelioration by spironolactone administration. ISSN: 1449-2288 Int J Biol Sci. Jun 6;11(8):892-900, 2015. FI: 4.5

4) Rodríguez-Romo R, Berman N, Gómez A, and Bobadilla N.A. Autor Corresponsal:Bobadilla NA. Epigenetic regulation in the acute kidney injury to chronic kidney disease transition (CKD). ISSN: 0085-2538 Nephrology 20:736-743, 2015. doi: 10.1111/nep.12521.FI: 2.1

5) Barrera-Chimal J., Pérez-Villalva R, Rodríguez-Romo R, Reyna J, Uribe N, Gamba G, Bobadilla N.A. Autor corrresponsal: Bobadilla NA- Spironolactone Prevents Chronic Kidney Disease caused by Ischemic Acute Kidney Injury Episode. ISSN: 0085-2538 Kidney Int 83(1):93-103, 2013. FI: 8.6

6) Barrera-Chimal J and Bobadilla N.A. Autor corrresponsal: Bobadilla NA. Response to: How to confirm the specific effect of spironolactone in chronic kidney disease caused by ischemic acute kidney injury? ISSN: 0085-2538 Kidney Int 84(2):415-416, 2013. FI: 8.6

Formación de Recursos Humanos

Tesis de Doctorado concluidas bajo mi dirección

Alumno: Roxana Rodríguez Romo
Grado: Doctorado en Ciencias Biomédicas 5 de mayo de 2016
Título: Implicación de la Angiotensina II en la Progresión a Enfermedad Renal Crónica (ERC) como consecuencia de la Lesión Renal Aguda
Lugar: Instituto de Investigaciones Biomédicas, UNAM.
Distinciones: Mención Honorífica

Posición Actual: Responable del Enlace Médico-Científico en Janssen

Alumno: Jonatan Barrera Chimal
Grado: Doctorado en Ciencias Biomédicas 9 de mayo de 2013
Título: Estudio de los mecanismos involucrados en la lesión renal aguda y su progresión a enfermedad renal crónica: diagnóstico y terapéutica
Lugar: Instituto de Investigaciones Biomédicas, UNAM.
Distinciones: Mención Honorífica, Premio a la mejor tesis doctoral, Premio Nacional de la Juventud, Medalla Dr. Gustavo Baz Prada, Nivel I del SNI

Tesis de Licenciatura concluidas bajo mi dirección:

Alumno: Arturo Gómez Romero
Grado: Licenciatura en Biología
Título: Alteraciones histológicas en la progresión a enfermedad renal crónica secundaria a un evento de lesión renal aguda.
Lugar: Facultad de Ciencias, UNAM, 7 abril de 2016

an apparent disturbance in acid–base homeostasis. However, it is not clear whether the association of unmeasured anions with mortality would differ between CKD patients with and without acidosis. As we stated in our paper, the association of the full anion gap with mortality appears to be independent of serum bicarbonate.

Souza *et al.*[1] also present biochemical evidence of possible unmeasured cation accumulation. Similarly, Parikh *et al.*[3] suggested unmeasured cations as one cause of a low anion gap in advanced CKD. However, Souza *et al.*[1] state that metabolic alkalosis is common among their patients with unmeasured cation accumulation. It is unclear as to what data support this claim, as the mean serum bicarbonate in this subgroup of patients was 23.9 ± 6.3 mEq/l. Finally, we agree that much remains to be learned about the nature of accumulated metabolites in CKD and their contribution to morbidity and mortality in this population.

1. Souza LE, de Queiroz REB, Libório AB. Unmeasured anions and cations in advanced chronic kidney disease. *Kidney Int* 2013; **84**: 413–414.
2. Abramowitz MK, Hostetter TH, Melamed ML. The serum anion gap is altered in early kidney disease and associates with mortality. *Kidney Int* 2012; **82**: 701–709.
3. Parikh C, Gyamlani G, Panlilio N *et al.* Unmeasured cations: probable cause of relatively low anion gap in chronic renal failure. *Ren Fail* 2001; **23**: 91–96.

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Kidney International (2013) **84,** 414–415; doi:10.1038/ki.2013.183

How to confirm the specific effect of spironolactone in chronic kidney disease caused by ischemic acute kidney injury?

To the Editor: Jonatan *et al.*[1] recently published a great study about the protective effect of spironolactone on chronic kidney disease (CKD) caused by ischemic acute kidney injury (AKI). The study showed that spironolactone administered either before or after ischemic kidney injury could prevent the subsequent CKD. However, we wondered whether CKD was prevented by the specific effect of spironolactone or just by alleviating the severity of AKI.

Both clinical observation and basic studies have demonstrated that even many aspects of renal function, such as serum levels of creatinine, are recovered after AKI; several other aspects of the kidney are permanently damaged and CKD develops.[2] In addition, the severity of AKI was associated with the subsequent development of CKD. In the study of Jonatan *et al.*,[1] the spironolactone was given before or immediately after AKI. If spironolactone could really prevent renal injury induced by ischemic reperfusion according to a previous study,[3] renal dysfunction might be less severe in the group with spironolactone administration. However, figure 1 in the study of Jonatan *et al.*[1] showed no significant difference of serum creatinine and proteinuria between both groups on day 10 after renal injury.

Serum creatinine on the first few days after ischemic renal injury was needed to clarify the consistency of the severity of AKI. Even though the creatinine levels are similar, the severity might be attenuated after spironolactone treatment because serum creatinine was not sensitive enough. To confirm the effect of spironolactone on CKD development, it might be administered 1 week after ischemic AKI, when most aspects of renal function are recovered, to see whether spironolactone could mitigate the risk factor of subsequent CKD such as the transforming growth factor-β pathway[4] or other alleged mechanisms.

1. Jonatan BC, Rosalba PV, Roxana RR *et al.* Spironolactone prevents chronic kidney disease caused by ischemic acute kidney injury. *Kidney Int* 2012; **83**: 93–103.
2. Basile DP, Deborah D, Kelly R *et al.* Renal ischemic injury results in permanent damage to peritubular capillaries and influences long-term function. *Am J Physiol Renal Physiol* 2001; **281**: F887–F899.
3. Mejia-Vilet JM, Ramirez V, Cruz C *et al.* Renal ischemia–reperfusion injury is prevented by the mineralocorticoid receptor blocker spironolactone. *Am J Physiol Renal Physiol* 2007; **293**: F78–F86.
4. Wu CF, Chiang WC, Lai CF *et al.* Transforming growth factor β-1 stimulates profibrotic epithelial signaling to activate pericyte-myofibroblast transition in obstructive kidney fibrosis. *Am J Pathol* 2013; **182**: 118–131.

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The Authors Reply: We thank Drs Chou and Lin for their observation[1] that allowed us to further expand the results of our findings. Previously, we showed that mineralocorticoid receptor blockade (MRB) or adrenalectomy prevented renal damage induced by a mild ischemic period (20 min).[2,3] In our recently published study,[4] rats were exposed to a more severe ischemic injury (45 min), and we observed that MRB before or even after the ischemic insult prevented chronic kidney disease (CKD) development, which was associated

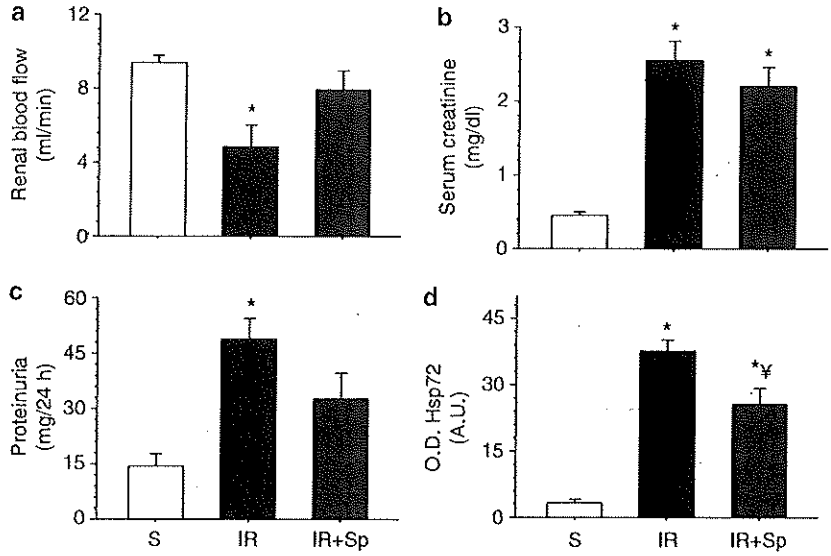

Figure 1 | Severe ischemic injury is partially prevented by prophylactic spironolactone administration. (**a**) Renal blood flow in the sham (white bar), bilateral renal ischemia (black bar), and rats receiving spironolactone (20 mg per kg of body weight) 3 days before ischemia (gray bar); (**b**) serum creatinine; (**c**) urinary protein excretion; and (**d**) urinary heat shock protein 72 (Hsp72). $^{*}P<0.05$ vs. the sham group and $^{¥}P<0.05$ vs. the ischemia–reperfusion (IR) untreated group. A.U., arbitrary units; O.D., optical density; S, sham; Sp, spironolactone.

with a reduction in acute kidney injury (AKI) severity, as was evidenced by a lower proteinuria 24 h after reperfusion (Figure 1a)[4]. Here, we added a new set of rats that underwent 45 min of bilateral ischemia and were studied after 24 h, as is shown in Figure 1. The prophylactic MRB only partially protected these animals as is exhibited by the reduction of levels of urinary heat shock protein 72 (Figure 1d).[5] These results emphasize that the degree of AKI was lower than that in untreated rats although serum creatinine was not corrected. In spite of the complete restoration of renal function observed after 10 days of ischemia, MRB was effective enough to prevent the increased signaling of inflammation and fibrosis pathways (Figure 1e and f)[4] that is not seen in ischemic untreated rats. Therefore, we believe that MRB not only reduced the severity of AKI but it also avoided other aldosterone detrimental effects. We agree with Drs Chou and Lin that it will be necessary to see the time course of these pathways to know with more precision the effects of aldosterone leading to long-term development of CKD. Indeed, this is a part of another ongoing project in our laboratory.

1. Chou YH, Lin SL. How to confirm the specific effect of spironolactone in chronic kidney disease caused by ischemic acute kidney. *Kidney Int* 2013; **84**: 415.
2. Mejia-Vilet JM, Ramirez V, Cruz C *et al.* Renal ischemia-reperfusion injury is prevented by the mineralocorticoid receptor blocker spironolactone. *Am J Physiol Renal Physiol* 2007; **293**: F78–F86.
3. Ramirez V, Trujillo J, Valdes R *et al.* Adrenalectomy prevents renal ischemia-reperfusion injury. *Am J Physiol Renal Physiol* 2009; **297**: F932–F942.
4. Barrera-Chimal J, Perez-Villalva R, Rodriguez-Romo R *et al.* Spironolactone prevents chronic kidney disease caused by ischemic acute kidney injury. *Kidney Int* 2013; **83**: 93–103.
5. Barrera-Chimal J, Perez-Villalva R, Cortes-Gonzalez C *et al.* Hsp72 is an early and sensitive biomarker to detect acute kidney injury. *EMBO Mol Med* 2011; **3**: 5–20.

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Kidney International (2013) **84,** 415–416; doi:10.1038/ki.2013.166

A more accurate replacement for the revised Schwartz equation: quadratic or Flanders metadata?

To the Editor: Dr Gao *et al.*[1] presented a new glomerular filtration rate (GFR) equation, the quadratic equation that outperformed the well-known revised Schwartz equation in predicting high GFR values. While we applaud the authors' well-designed and analyzed study, we would appreciate it if the following point could be clarified.

The main limitation of the revised Schwartz equation, i.e., lack of an age-dependent adjustment factor, was first highlighted in a meta-analysis conducted by Pottel *et al.*[2] In their study, Pottel *et al.* challenged the value of k in the revised Schwartz equation for healthy children, and developed an age-dependent modification for k ($k' = 0.0414 \times \ln(\text{Age}) + 0.3018$). This modified version was shown to overcome the inaccuracy of the revised Schwartz equation in estimating GFR in children with normal renal function. Correspondingly, the authors suggested using this modified version of the Schwartz equation (named the Flanders metadata equation) in 'all

Spironolactone prevents chronic kidney disease caused by ischemic acute kidney injury

Jonatan Barrera-Chimal[1,2], Rosalba Pérez-Villalva[1,2], Roxana Rodríguez-Romo[1,2], Juan Reyna[1,2], Norma Uribe[3], Gerardo Gamba[1,2] and Norma A. Bobadilla[1,2]

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Acute kidney injury (AKI) has been recognized as a risk factor for the development of chronic kidney disease (CKD). Aldosterone has a critical role in promoting renal injury induced by ischemia. Here, we evaluated whether spironolactone administered before or after AKI caused by ischemia protects against CKD. In the first set of experiments, Wistar rats underwent a sham operation without or with prior spironolactone treatment, or underwent 45 minutes of bilateral renal ischemia without or with spironolactone treatment before ischemia and assessed over 270 days. The second set of rats received low (20 mg/kg) or high (80 mg/kg) doses of spironolactone at three different times after the sham operation or bilateral renal ischemia and were assessed after 90 days. Untreated animals developed CKD following ischemia-induced AKI as characterized by a progressive increase in proteinuria, renal dysfunction, podocyte injury, glomerular hypertrophy, and focal sclerosis. This was associated with increased oxidative stress, an upregulation of tumor growth factor (TGF)-β, followed by upregulation of the TGF-β downstream effectors phospho-Smad3, collagen I, fibronectin, and proinflammatory cytokines. Treatment with spironolactone either before or after ischemia prevented subsequent CKD by avoiding the activation of fibrotic and inflammatory pathways. Thus, spironolactone may be a promising treatment for the prevention of AKI-induced CKD.

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KEYWORDS: inflammatory cytokines; mineralocorticoid receptor blockade; oxidative stress; TGF-β pathway; tubular proliferation

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Ischemic kidney injury is the primary cause of acute kidney injury (AKI) in hospitalized patients and is associated with high morbidity and mortality rates. In addition, the incidence of AKI has significantly increased in the past 15 years owing to an aging general population and the increased prevalence of obesity, diabetes, and hypertension, which predispose patients to an AKI event.[1–5] AKI occurs in approximately 15% of hospitalized patients and in up to 40–60% of intensive care unit patients.[6,7]

For many years, it was commonly thought that patients surviving episodes of AKI recover their renal function without further consequences. However, recent evidence based on epidemiological observations in patients who suffer from AKI strongly suggests otherwise. AKI has thus been proposed to be a risk factor for developing chronic kidney disease (CKD) and, in particular, for promoting the transition of CKD to end-stage renal disease.[8–11] In support of this hypothesis, the probability of developing CKD or end-stage renal disease over time is proportional to the severity and duration of the AKI event.[8,12,13] Moreover, of great concern is the recent evidence demonstrating that 6.6% of AKI patients who had a complete renal function recovery exhibited a greater risk of death and *de novo* CKD after 2–4 years of follow-up.[12] Until now, however, the progression of AKI to CKD in rats with two intact kidneys, which would allow elucidating the mechanisms causing AKI to progress to CKD, has been rarely explored. Such a model would also be beneficial for identifying pharmacological interventions to prevent injury due to AKI and/or stop the development of CKD and end-stage renal disease.

Several studies involving experimental models have demonstrated that aldosterone has an important role in the physiopathology of renal injury induced by ischemic process, including acute and chronic cyclosporine A nephrotoxicity and ischemia/reperfusion (I/R).[14–21] Accordingly, we have shown that prophylactic spironolactone treatment[16] or adrenal gland removal[17] completely prevents AKI in rats undergoing bilateral renal ischemia. Furthermore, spironolactone administration at different postischemia intervals prevents or reduces functional and structural renal injury,[22] suggesting that aldosterone is a key molecule in mediating ischemic renal

injury and that mineralocorticoid receptor (MR) antagonism, even after ischemia, is a helpful strategy to prevent AKI.

In this study, we characterized a model of CKD induced by a single episode of ischemic AKI and the molecular mechanisms that lead to the development of CKD. Our data reveal that spironolactone administration before or after the ischemic insult is a useful strategy to prevent or reduce the development of CKD.

RESULTS

We have previously shown that, after 24 h of bilateral ischemia, rats develop severe renal dysfunction and tubular injury.[16] Figure 1 shows that renal dysfunction induced by ischemia was completely resolved after 9 days of reperfusion, similar to conditions that often occur in clinical settings. Urinary protein excretion was significantly elevated after 24 h of reperfusion in rats subjected to ischemia and continues to progressively decline until normal values were reached after 6 days of reperfusion (Figure 1a). Proteinuria in spironolactone-pretreated rats was 50% lower than the proteinuria in the untreated group, and normal levels were reached faster. Consequently, the rats undergoing ischemia recovered renal function after 10 days, as demonstrated by normal values of renal blood flow, serum creatinine, and creatinine clearance (Figure 1b–d). Despite the complete improvement in renal function, interleukin (IL)-6 mRNA and phosphorylated Smad3 levels were significantly higher than those in the sham-operated group. Cytokine upregulation was not observed in spironolactone-pretreated rats (Figure 1e–f). These results suggest that although renal function recovered, proinflammatory and profibrotic pathways remained active. These pathways may be able to perpetuate renal injury, which can lead to CKD, a situation that did not occur in spironolactone-pretreated animals.

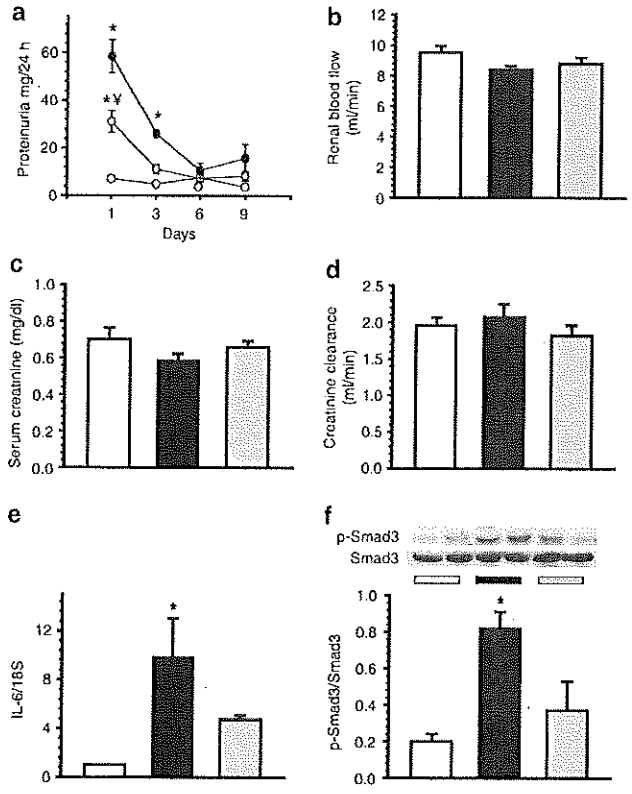

Figure 1 | Renal dysfunction induced by ischemia was completely resolved after 10 days of reperfusion, but proinflammatory and profibrotic cytokines remained enhanced. (**a**) Urinary protein excretion 1, 3, 6, and 9 days after reperfusion, in the sham (o), bilateral renal ischemia (•), and ischemia + Sp pretreatment (•) groups. At 10 days after reperfusion, the rats were killed, and renal function was determined. (**b**) Mean renal blood flow, (**c**) serum creatinine, and (**d**) creatinine clearance. (**e**) Interleukin (IL)-6 mRNA levels were measured in the renal cortex. (**f**) The upper insets show representative autoradiographs from the phosphorylated-Smad3 (p-Smad3) and Smad3 western blot analysis for sham (white bars), bilateral renal ischemia (black bars), and ischemia + Sp pretreatment (gray bars) groups. The lower graphs display the corresponding densitometric analysis. $^{*}P < 0.05$ vs. the sham group and $^{¥}P < 0.05$ vs. the ischemia group.

Ischemic insult leads to progressive renal dysfunction that can be prevented with spironolactone pretreatment

We assessed whether an AKI episode induced by ischemia leads to CKD. Because we have previously shown that I/R injury is prevented by spironolactone pretreatment or adrenal gland removal,[16,17] we evaluated whether spironolactone pretreatment before ischemia also prevents CKD development. There was considerable mortality in the A-to-C group in the first 10 days after ischemia (57%), and survival was markedly improved by spironolactone pretreatment (15%) (Figure 2a). Proteinuria was assessed every 30 days. The animals that survived the ischemic insult developed a progressive increase in proteinuria, from 20.2 ± 1.5 mg/day at 30 days to 164.8 ± 11.3 mg/day at 270 days. This increase was not observed in the A-to-C + Sp group (9.2 ± 1.0 mg/day at 30 days and 27.1 ± 2.0 mg/day at 270 days) (Figure 2b). Despite the similar body weights of all rats at the beginning of the study, the A-to-C group exhibited a lower average body weight at the end of the study (583 ± 16.3 g) than did the S, Sp, and A-to-C + Sp groups (752 ± 28.3, 729 ± 19.2, and 721 ± 11.8 g, respectively). After 9 months, the A-to-C group developed renal dysfunction that was characterized by a significant reduction in renal blood flow and creatinine clearance (Figure 2c and d). Interestingly, the A-to-C + Sp group failed to develop renal dysfunction. As shown in Figure 2e, at the end of the study, the mean arterial pressure was similar among the groups. Therefore, the renal injury observed in the rats that developed CKD was not due to hypertension. In agreement with these findings, the rats that developed CKD exhibited an increase in the levels of urinary kidney injury molecule-1, an effect that was not observed in the A-to-C + Sp group (Figure 2f).

Ischemic insult leads to severe renal structural injury: prevention by spironolactone pretreatment

Representative light microscopy sections from rat kidneys stained with periodic acid–Schiff are shown in Figure 3a–d.

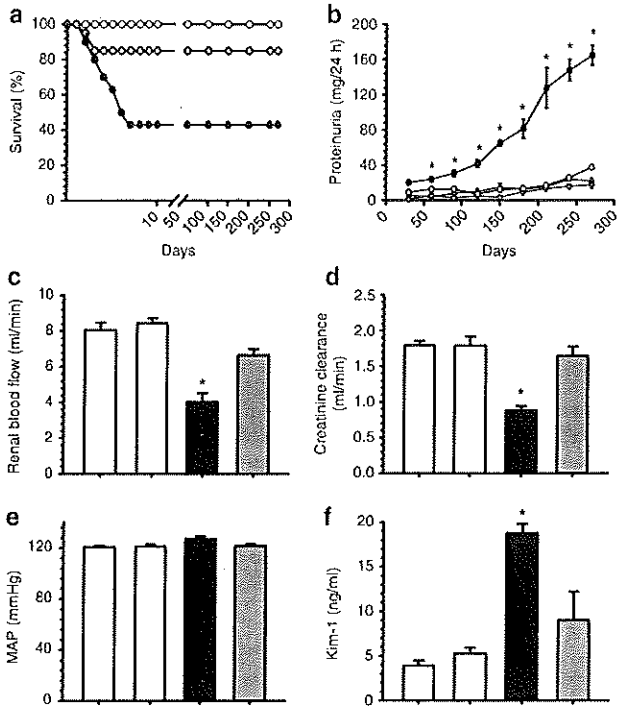

Figure 2 | Acute kidney injury leads to the development of chronic kidney disease (CKD), which can be prevented by spironolactone pretreatment. Four groups were included: sham surgery (S), $n = 9$; rats receiving spironolactone 3 days before sham surgery (20 mg/kg per day), $n = 9$ (Sp); rats undergoing renal bilateral ischemia, $n = 28$ (A-to-C); and rats receiving spironolactone 3 days before bilateral ischemia, $n = 13$ (A-to-C + Sp). (**a**) The survival rate in the A-to-C group (black circles) was 43%, compared with 85% in the A-to-C + Sp group (gray circles) and 100% in the sham and Sp group (overlaid white circles). (**b**) Urinary protein excretion was measured every 30 days during follow-up: sham (▽), Sp (△), A-to-C (•), and A-to-C + Sp (○). At the end of the experimental period, (**c**) renal blood flow, (**d**) creatinine clearance, (**e**) mean arterial pressure, and (**f**) urinary kidney injury molecule-1 (Kim-1) levels were determined in the sham (white bars), Sp (white bars), A-to-C (black bars), and A-to-C + Sp groups (gray bars). *$P < 0.05$ vs. the S and Sp groups.

Rats undergoing ischemia exhibited severe structural alterations characterized by glomerular hypertrophy, glomerulosclerosis, cast formation, severe tubular dilation, and tubulointerstitial fibrosis. By contrast, the A-to-C + Sp group exhibited glomerular and tubular architecture similar to that of control rats. These findings were corroborated by quantification of the number of dilated tubules, percentage of glomerulosclerosis, and glomerular diameter size (Figures 3 and 4). Tubular dilation was present in 42.3 ± 5.0% of the total tubules in the A-to-C group, whereas only 13.1 ± 2.7% of the A-to-C + Sp group displayed dilation ($P < 0.01$). Similarly, the A-to-C group exhibited a significantly higher glomerulosclerosis percentage (10.0 ± 4.4%) than the S and A-to-C + Sp groups (0% and 0.4 ± 0.4%, respectively).

The degree of glomerular hypertrophy was evaluated by measuring glomerulus diameter and by generating a distribution of glomerular size. Figure 4a shows the normal diameter distribution of the control group, wherein most of the glomeruli were in the range of 101–125 μm (38.3%), and only a minor proportion was found in the ranges of 76–100 μm (19.5%) and 126–150 μm (27.5%). The histogram for the control group exhibits a typical bell-shaped Gaussian distribution, as we have shown previously.[21] By contrast, in the A-to-C group, the glomerular diameter distribution was shifted to the right, reflecting glomerular hypertrophy. Accordingly, 43.3% of the glomeruli were >151 μm in diameter. In addition, a lower proportion of glomeruli were found in the diameter ranges of 101–125 μm (20.4%), 76–100 μm (8.3%), and 50–75 μm (0%). All of these differences were statistically significant according to contingency analysis, as shown in Figure 4b. The glomerular size distribution of the A-to-C + Sp group was similar to that of the control group, but not to that of the A-to-C group (Figure 4c), indicating that glomerular hypertrophy was nearly absent. In agreement with these findings, renal hypertrophy in the A-to-C group was also evidenced by a significant elevation in the mean kidney weight (2.5 ± 0.2 g) compared with the S and Sp groups (1.6 ± 0.1 and 1.6 ± 0.1 g, respectively); this increase was not observed in the A-to-C + Sp group (1.5 ± 0.1 g).

Transmission electron microscopy of rat kidneys with CKD revealed ultrastructural alterations that included microvillus degeneration and effacement and detachment of podocyte foot processes (Figure 4e). These alterations were rarely observed in the A-to-C + Sp group (Figure 4f). Furthermore, an extensive tubulointerstitial area was affected by fibrosis in the A-to-C group (Figure 5a and b), but a lower extent of fibrosis was observed in the A-to-C + Sp group (Figure 5c and d). These observations were confirmed by the morphometric analysis represented in Figure 5e. The A-to-C group exhibited fibrosis in 44.8 ± 16.0% of the tubulointerstitial area, compared with 18.7 ± 4.5% in the A-to-C + Sp group; this difference was significant.

Tubular dilation is due in part to cellular proliferation

To determine whether the severe tubular dilation observed in the A-to-C group was associated with tubular cell proliferation, immunohistochemical analysis of proliferating cell nuclear antigen (PCNA) was performed. The A-to-C group exhibited considerable tubular cell proliferation as demostrated by the number of PCNA + cells, an effect that was almost absent in the A-to-C + Sp group (Figure 6a–c). These findings were confirmed by calculating the percentage of nuclei that stained positive for PCNA (Figure 6d). The positive cells were primarily located in the dilated tubules, suggesting that enhanced cell proliferation is promoting tubular dilation in the A-to-C group. This assumption is also supported by a significant correlation between the percentage of dilated tubules and the percentage of PCNA + cells (Figure 6e, $r^2 = 0.87$).

Tubulointerstitial fibrosis is mediated by TGF-β pathway activation

The role of the tumor growth factor (TGF)-β pathway in promoting tubulointerstitial fibrosis was also evaluated.

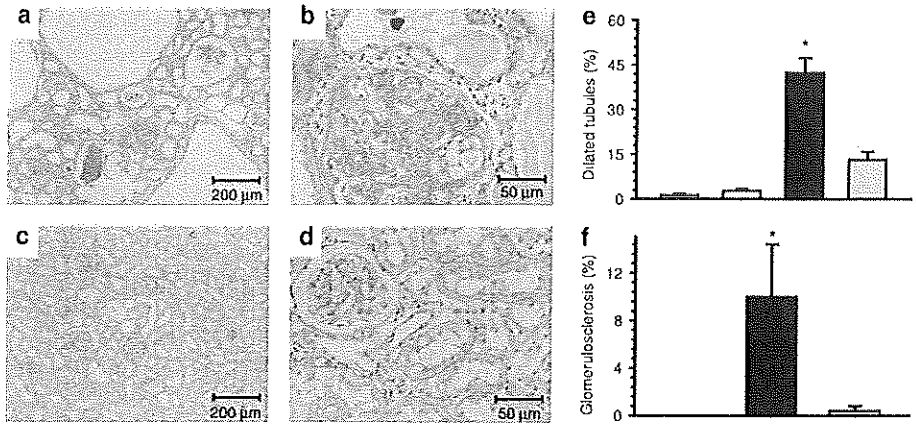

Figure 3 | An acute kidney injury (AKI) episode leads to severe structural damage, which can be prevented by Sp pretreatment. (**a**, **b**) Representative images of periodic acid–Schiff (PAS)-stained sections from the A-to-C group, and (**c**, **d**) sections from the A-to-C + Sp group. (**e**) The percentage of dilated tubules was quantified by counting the number of dilated tubules as a proportion of the total number of tubules. (**f**) Glomerulosclerosis percentage for the sham (white bars), Sp (second set of white bars), A-to-C (black bars), and A-to-C + Sp groups (gray bars). *$P<0.05$ vs. the S and Sp groups.

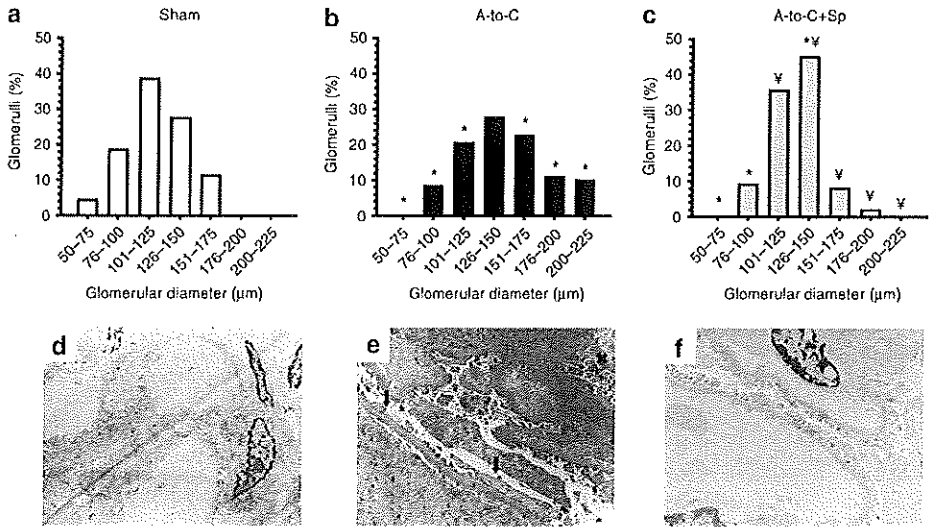

Figure 4 | Glomerular hypertrophy and ultrastructural lesions in rats with chronic kidney disease (CKD). (**a**) The glomerular diameter distribution is represented in white bars for the sham group, (**b**) in black bars for the A-to-C group, and (**c**) in gray bars for the A-to-C + Sp group. Representative transmission electron micrographs are shown in (**d**) the sham group, (**e**) the A-to-C group, and (**f**) the A-to-C + Sp group. Black arrows indicate foot process fusion, and asterisks indicate foot process detachment. Original magnification: ×6,300. *$P<0.05$ vs. the S group and $^{¥}P<0.05$ vs. the A-to-C group.

The A-to-C group exhibited a significant twofold elevation in TGF-β mRNA levels. This increase was not observed in the A-to-C + Sp group (Figure 7a). To assess whether this pathway was efficiently activated, the renal levels of downstream effectors of the TGF-β pathway, including phosphorylated Smad3, fibronectin, collagen I, and α-smooth muscle actin (α-SMA), were measured by western blot analysis as shown in Figure 7b–f. The levels of all of these proteins were significantly elevated in the rats that developed CKD. Intriguingly, the activation of the TGF-β pathway was completely prevented in the A-to-C + Sp group. Recent studies have shown that when Smad2 is phosphorylated, it can act as an antifibrotic modulator of the TGF-β pathway.[23] We found that the A-to-C + Sp group, which exhibited less tubulointerstitial fibrosis, also displayed a significant elevation in phospho-Smad2 levels.

Renal injury induced by an ischemic insult is also mediated by increased oxidative stress

Urinary H_2O_2 excretion in the A-to-C group was significantly elevated compared with the S and Sp groups (64.1 ± 7.5 vs. 10.4 ± 1.1 and 5.9 ± 1.0 nmol/min, respectively) (Figure 8a). However, the elevation in urinary H_2O_2 excretion was abrogated in the A-to-C + Sp group (14.8 ± 2.5 nmol/min). In addition, catalase activity was significantly reduced in the A-to-C group (Figure 8b), and this effect was almost entirely prevented in the

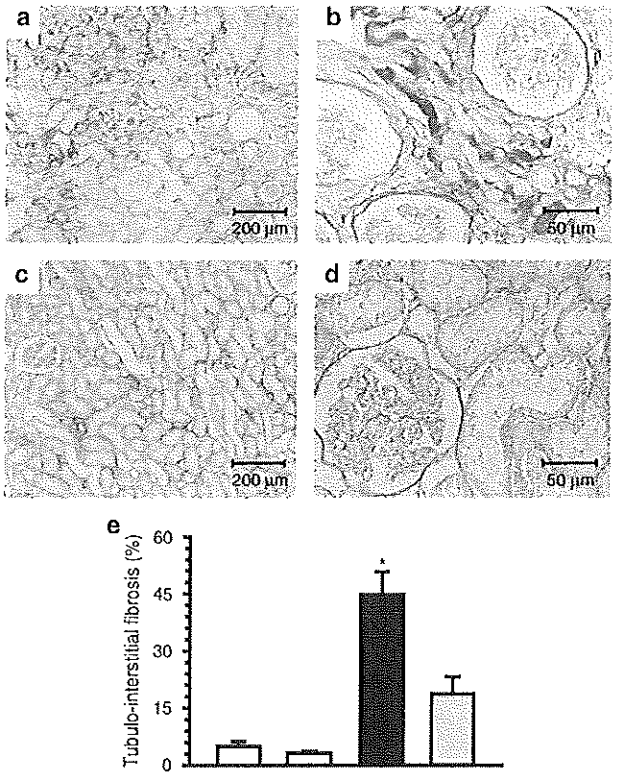

Figure 5 | Tubulointerstitial fibrosis in rats with chronic kidney disease (CKD). (**a**, **b**) Representative light micrographs after Sirius red staining showing the presence of fibrosis (in red) from the A-to-C group, and (**c**, **d**) micrographs from the A-to-C + Sp group. (**e**) The percentage of tubulointerstitial fibrosis in each of the four groups at the end of the 270-day experiment was quantified by morphometric analysis for sham (white bars), Sp (second set of white bars), A-to-C (black bars), and A-to-C + Sp groups (gray bars). $^{*}P < 0.05$ vs. the S and Sp groups.

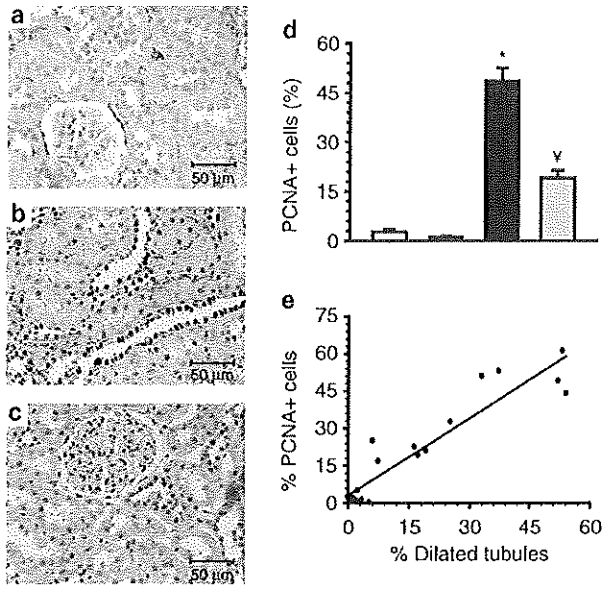

Figure 6 | Tubular cell proliferation as assessed by proliferating cell nuclear antigen (PCNA) immunohistochemistry. To evaluate whether epithelial cells were proliferating and provoking tubule dilation, immunohistochemistry for PCNA was performed in kidney sections from the four groups. (**a**) Representative images from the sham, (**b**) A-to-C, and (**c**) A-to-C + Sp groups. (**d**) The percentage of PCNA + cells in the sham (white bars), Sp (second set of white bars), A-to-C (black bars), and A-to-C + Sp groups (gray bars). (**e**) The correlation between the percentage of PCNA + cells and the percentage of dilated tubules ($r^2 = 0.87$). $^{*}P < 0.05$ vs. the S and Sp groups, and $^{¥}P < 0.05$ vs. the A-to-C group.

A-to-C + Sp group. No differences in glutathione peroxidase activity were observed among the groups (Figure 8c).

Inflammatory cytokine upregulation is involved in ischemia-induced CKD

Tumor necrosis factor alpha (TNF-α), monocyte chemotactic protein-1 (MCP-1), and IL-6 mRNA and protein levels were assessed by real-time reverse transcription–polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. As shown in Figure 9, the mRNA levels of these cytokines were upregulated in the A-to-C group by 1.6-fold for TNF-α and more than 13-fold for MCP-1 and IL-6, compared with the S group. By contrast, the A-to-C + Sp group exhibited mRNA levels similar to the control group. These observations were corroborated at the protein level; a similar pattern was observed in the enzyme-linked immunosorbent assay results.

CKD induced by AKI was also reduced by spironolactone administration after the ischemic insult

Spironolactone administered at 0 or 1.5 h after the ischemic insult prevented the development of proteinuria compared with the untreated A-to-C group either at low or high doses (Figure 10a and d). A low dose of spironolactone administered 3 h after ischemia was unable to prevent, but did significantly reduce, the progressive elevation of proteinuria (Figure 10a). These findings were not associated with significant changes in renal blood flow (Figure 10b and e) or creatinine clearance (Figure 10c and f), probably because 3 months is an early stage of the CKD, often exhibiting proteinuria, without renal dysfunction, as was observed after a longer period of observation after similar ischemia (Figure 2). Despite the lack of significant differences among these groups, spironolactone-treated animals exhibited better renal function than the ischemic group.

At 3 months after inducing bilateral renal ischemia, the untreated A-to-C group, compared with the sham-operated group, exhibited morphological alterations, such as tubular dilation, cast formation, glomerular hypertrophy, and extensive tubulointerstitial fibrosis (Supplementary Figure S1 online). All of these changes were reduced in the animals treated with a low dose of spironolactone (Supplementary Figure S2 online) and were prevented in animals treated with a high dose of spironolactone (Supplementary Figure S3 online). In fact, glomerular hypertrophy (measured as the distribution of glomerular diameters) was prevented in the groups that received a low dose of spironolactone at 0 h

(Figure 11c) or high dose at either 0 or 1.5 h after renal bilateral ischemia (Figure 11f and g, respectively). Although kidney changes were not completely prevented, these renoprotective effects of spironolactone were also observed with a low dose at 1.5 and 3 h after ischemia (Figure 11d and e, respectively). Morphometric analyses demonstrated that, 3 months after inducing ischemia, the untreated rats exhibited fibrotic damage in 33.3% of their tubulointerstitium compared with damage in 3.7% of the tubulointerstitium in the sham-operated rats (Supplementary Figure S1 online). In contrast, a low dose of spironolactone administered either at 0 and 1.5 h post ischemia reduced the tubulointerstitial fibrosis to 14.8% and 15.1%, respectively, and the 3-h administration reduced the fibrosis to a lesser extent, 20.9% (Supplementary Figure S2 online). A high dose of spironolactone at 0 or 1.5 h post ischemia was more effective in reducing the area affected by tubulointerstitial fibrosis to 11.8% and 7.8%, respectively (Supplementary Figure S3 online).

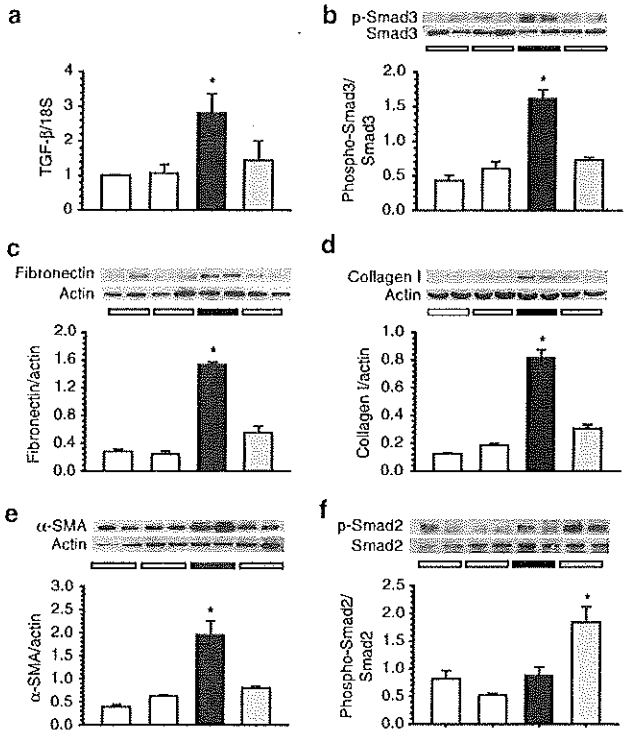

Figure 7 | Association of renal fibrosis with the tumor growth factor (TGF)-β pathway activation. (a) TGF-β mRNA levels were quantified by real-time reverse transcription–polymerase chain reaction (RT-PCR). Densitometric analysis of the western blots was performed for (b) phospho-Smad3 (p-Smad3), (c) fibronectin, (d) collagen I, (e) α-smooth muscle actin (α-SMA), and (f) phospho-Smad2 in the sham (white bars), Sp (second set of white bars), A-to-C (black bars), and A-to-C + Sp groups (gray bars). In each panel, the upper insets depict representative blots of the corresponding proteins. *$P < 0.05$ vs. the S and Sp groups.

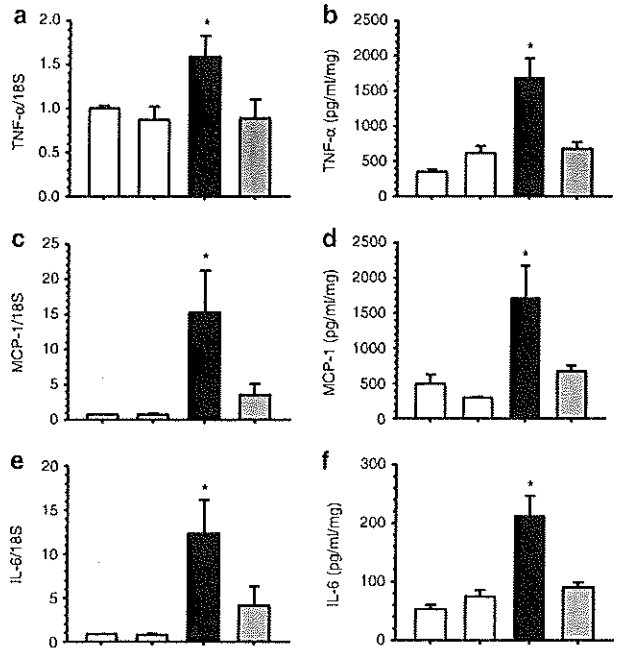

Figure 9 | The contribution of the inflammatory response to chronic kidney disease (CKD) development. (a) TNF-α, (c) MCP-1, and (e) IL-6 mRNA levels measured in total kidney RNA extracted from the sham (white bars), Sp (second set of white bars), A-to-C (black bars), and A-to-C + Sp groups (gray bars). (b) TNF-α, (d) MCP-1, and (f) IL-6 protein levels quantified by enzyme-linked immunosorbent assay (ELISA) in tissue kidney homogenates. *$P < 0.05$ vs. the S and Sp groups.

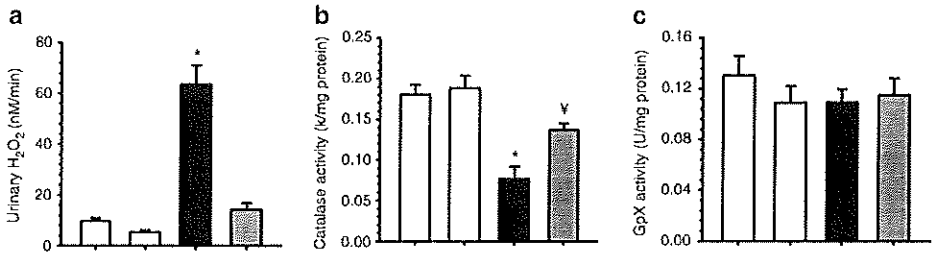

Figure 8 | The contribution of oxidative stress to chronic kidney disease (CKD) development and prevention by spironolactone pretreatment. (a) Urinary hydrogen peroxidase (H_2O_2) excretion after 270 days of follow-up. (b) Catalase activity and (c) glutathione peroxidase (Gpx) activity in the sham (white bars), Sp (second set of white bars), A-to-C (black bars), and A-to-C + Sp groups (gray bars). *$P < 0.05$ vs. the S and Sp groups; ¥$P < 0.05$ vs. the A-to-C group.

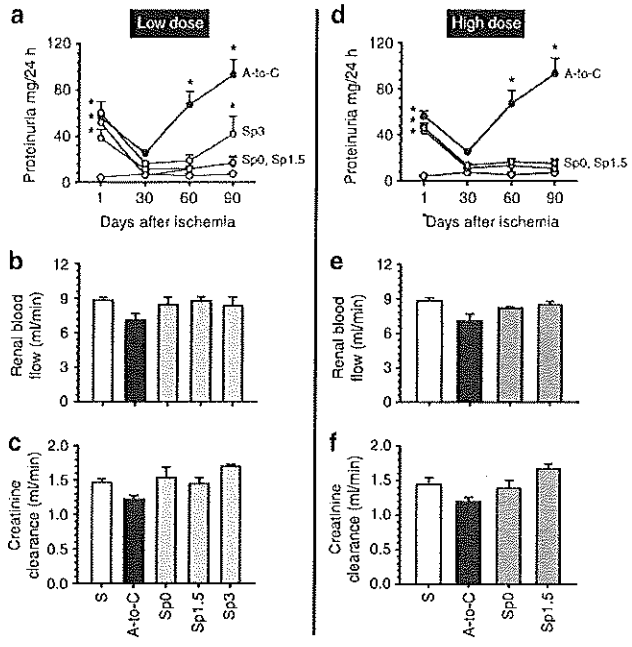

Figure 10 | Spironolactone administration after the ischemic insult prevents the development of proteinuria. (**a**) Urinary protein excretion at 1, 30, 60, and 90 days in sham (white circles), in A-to-C (black circles), and in rats receiving Sp (20 mg/kg) at 0, 1.5, or 3 hours after ischemia (gray circles) or (**d**) treated with a high dose of Sp (80 mg/kg) (dark gray circles) at 0 or 1.5 h after bilateral ischemia. After 90 days of follow-up, (**b**, **e**) renal blood flow and (**c**, **f**) creatinine clearance were determined in the sham group (white bars), A-to-C group (black bars), and the groups receiving a low dose of Sp (gray bars) at 0, 1.5, or 3 h after ischemia or a high dose of Sp (dark gray bars) at 0 or 1.5 h after bilateral ischemia. *$P < 0.05$ vs. sham-operated rats.

Molecular markers of renal fibrosis and inflammation also provided evidence of the protection conferred by spironolactone. The upregulation of TGF-β mRNA levels was prevented or reduced by a low or high dose of spironolactone administered at 0 or 1.5 h after ischemia (Figure 12a and e). However, increased phospho-Smad3 levels remained elevated in rats treated with a low dose, but not with a high dose, of spironolactone (Figure 12b and f). The renoprotective effect of spironolactone was also associated with the prevention of α-SMA and MCP-1 upregulation (Figure 12c–h).

DISCUSSION

In this study, we characterized a rat model of CKD induced by a single episode of AKI. We observed that an episode of AKI was resolved in surviving animals within a period of 10 days. However, although physiological parameters returned to normal values, activation of proinflammatory and profibrotic signals persisted. During the following months, progressive deterioration of renal function and renal structures was observed in rats surviving AKI, leading to the development of CKD. Thus, this model resembles what is currently believed to occur in the clinical setting.[11–13,24–27] Patients who survive an episode of AKI and apparently recover renal function may develop CKD later in life.[8,24] In this study, we observed that spironolactone administration before or after the ischemic insult prevented or significantly diminished the severity of an AKI episode, without signs of proinflammatory or profibrotic activation after 10 days of ischemia. Thus, spironolactone pretreatment resulted in a reduction in rat mortality and in the prevention of CKD. These observations demonstrate the relevance of the prevention of AKI episodes to a reduction in the prevalence of CKD.

CKD development in rats surviving AKI was characterized by a progressive increase in urinary protein excretion, renal dysfunction, glomerular hypertrophy, severe tubular dilation, tubulointerstitial fibrosis, and podocyte injury. Our study supports the hypothesis that an ischemic insult is sufficient to lead to progressive CKD in the rat, despite apparent recovery from the AKI episode. Because it was possible that CKD resulted from irreversible renal artery damage caused by the clamping, a group of rats was studied after 10 days of ischemia, confirming that this was not the case. After 10 days, renal function returned to normal values. Although renal histopathology was not analyzed at this point, we have previously reported that after 3 days of I/R, the tubular epithelium had almost recovered its normal structure.[28,29] However, in this study, we found that profibrotic and inflammatory cytokines remained at high levels despite a return to normal renal function values. In addition, cytokines such as TNF-α and IL-1β, IL-6, IL-12, IL-15, IL-18, and IL-32 are known to be induced as a result of the enhanced leukocyte activation and leukocyte–endothelial adhesion observed after I/R. Moreover, the renal tubular epithelium may generate proinflammatory cytokines such as TNF-α, IL-6, IL-1β, and TGF-β.[30–33] It is important to note that Basile *et al.*[34,35] and Hörbelt *et al.*[36] have demonstrated a persistent reduction in vascular density after I/R that maintains a continuous hypoxic and inflammatory state. Furthermore, Conger *et al.*[37] demonstrated that the postischemic kidney does not properly autoregulate the blood flow. All of these adverse conditions perpetuate continuous cycles of hypoxic damage and inflammation that injure the surrounding tissues and eventually lead to CKD, as has been clearly discussed by Bedfoord *et al.*[38] In agreement with all these findings, we found that, after 9 months, the rats that developed CKD exhibited greater levels of the proinflammatory cytokines compared with the rats that were pretreated with spironolactone. Interestingly, MCP-1 upregulation observed 3 months after ischemia was also prevented when spironolactone was administered after the insult. Our results suggest that the beneficial results of spironolactone in preventing chronic inflammation are a result of its ability to attenuate I/R-induced acute inflammation.

The CKD induced by an AKI episode was characterized by a progressive enhancement in urinary protein excretion and renal dysfunction without changes in mean arterial pressure.

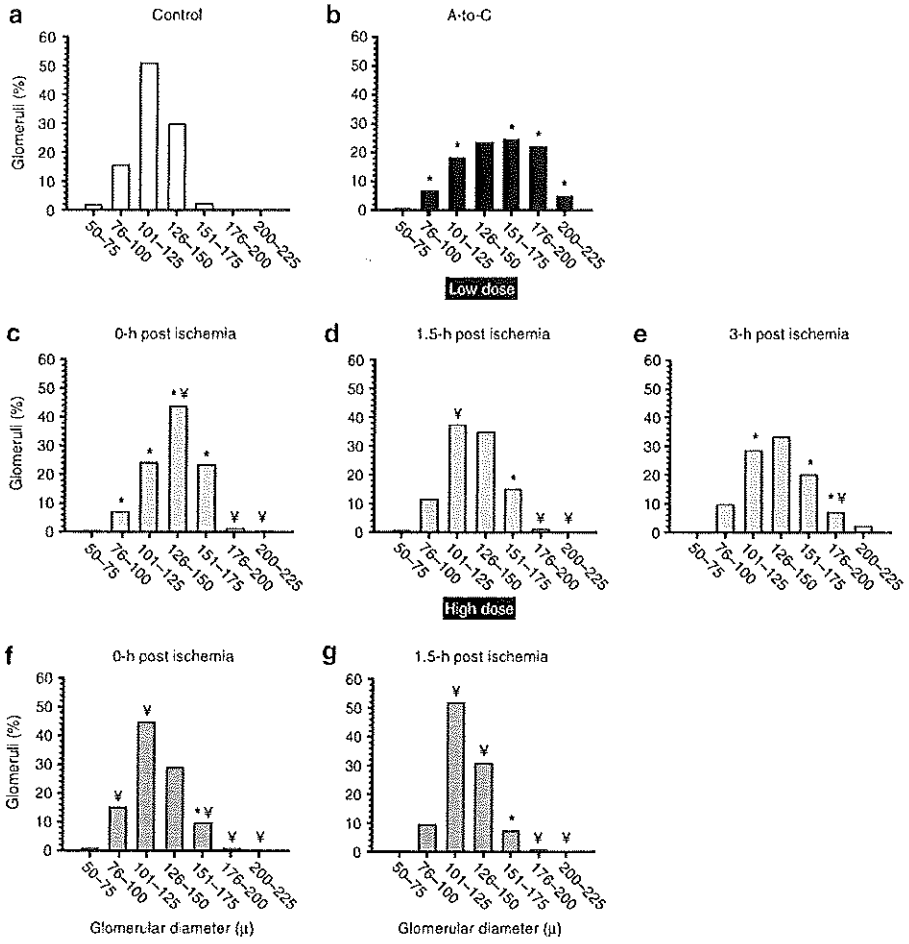

Figure 11 | Glomerular injury is prevented or reduced by spironolactone administration after renal ischemia. Glomerular diameter distribution in sham (white bars), A-to-C (black bars), low spironolactone dose (gray bars), and high spironolactone groups (gray dark bars). $^{*}P<0.05$ vs. the S group and $^{¥}P<0.05$ vs. the A-to-C group.

These conditions provide us with an experimental model that dissects the detrimental effect of an AKI episode on renal function and structure without changes in blood pressure. Accordingly, the animals that suffered CKD exhibited severe structural injury. Glomerular hypertrophy and glomerulosclerosis were observed 9 months after inducing ischemia. These alterations were also associated with ultrastructural changes. It is plausible that this glomerular injury is a consequence of the endothelial injury and the endothelial-to-mesenchymal transition that occurred after the ischemia.[34,35] Interestingly, in this study, we found that spironolactone administration both before and after renal ischemia prevented the transition of AKI to CKD.

Aldosterone binding to the MR has been suggested to influence endothelial function and vascular tone.[17,19,39,40] Supporting this finding, mice overexpressing MR in the endothelium exhibit altered vascular tone,[40] and aortic wall thickness has been reported in patients with primary hyperaldosteronism.[41] Consistent with this result, aldosterone infusion in mice reduces the expression of glucose-6-phosphate dehydrogenase (G6PDH), a key enzyme in maintaining the balance between nitric oxide and reactive oxygen species production.[42] Therefore, aldosterone has been proposed as a risk factor of vascular injury.[43] The mechanisms involved, however, have not been clearly elucidated. In this regard, we have previously demonstrated that MR antagonism precludes the characteristic hypoperfusion and oxidative stress induced by I/R, suggesting that aldosterone has a pivotal role in mediating renal dysfunction.[16,22] It is possible that the endothelium and the renal plasma flow of spironolactone-treated rats in this study was minimally affected as a consequence of the I/R injury; therefore, the glomerular structure remained unaffected in the long term.

Tubulointerstitial fibrosis is a common feature of CKD progression (for a review, see Rodriguez-Iturbe and Garcia[44]). In fact, severe tubular dilation and an extensive area affected by tubulointerstitial fibrosis were found in the A-to-C group. These results suggest that the tubulointerstitium is more susceptible to the effects of an AKI episode and contributes to the progression and severity of CKD in rats suffering from AKI. Accordingly, the severity of tubular damage reportedly exhibits a more significant correlation

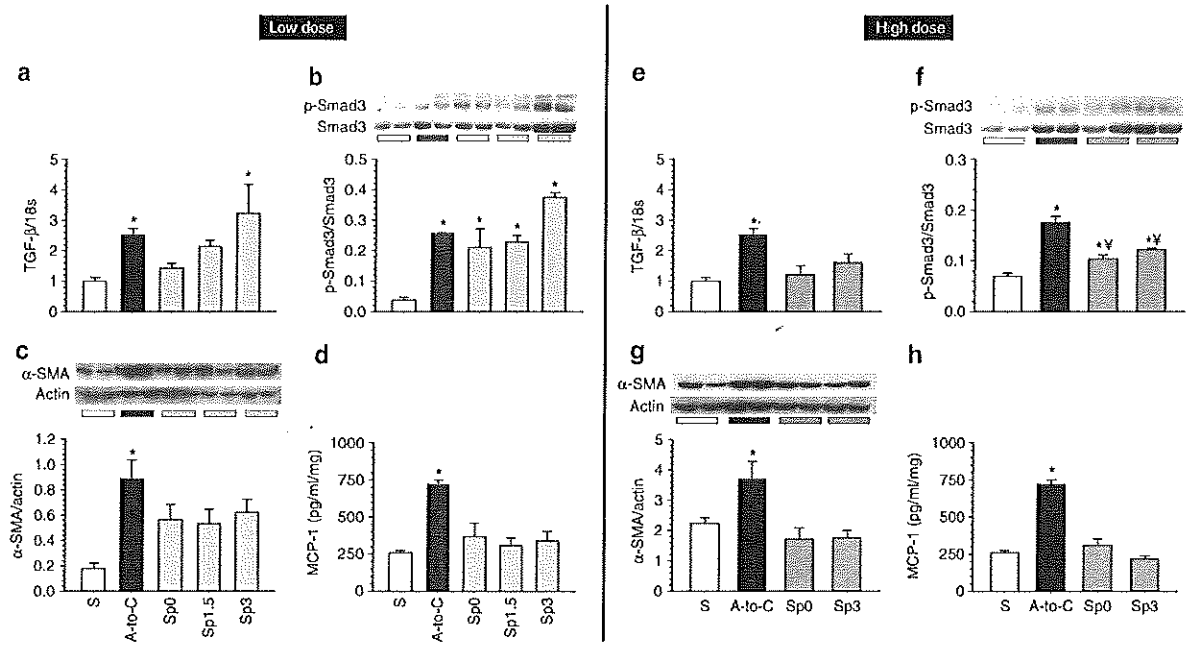

Figure 12 | Tumor growth factor (TGF)-β and inflammation pathways in rats treated with spironolactone after the ischemic insult. (**a**, **e**) TGF-β mRNA levels quantified by real-time reverse transcription-polymerase chain reaction (RT-PCR). Densitometric analysis of the western blots performed for (**b**, **f**) p-Smad3 and (**c**, **g**) α-SMA. (**d**, **h**) MCP-1 protein levels quantified in renal tissue by enzyme-linked immunosorbent assay (ELISA). Sham (white bars), A-to-C (black bars), low Sp dose (gray bars), and high Sp dose (dark gray bars). $^{*}P<0.05$ vs. the S group and $^{¥}P<0.05$ vs. the A-to-C group.

with the reduction in creatinine clearance than with glomerular injury scores.[45,46] These tubular defects were associated with an increase in tubular cell proliferation, as demonstrated by the significant increase in PCNA + staining and by the strong correlation with tubular dilation ($r^2 = 0.87$). In this study, however, the location of PCNA + cells in the epithelium of the dilated tubules strongly suggests that the tubular dilation observed in the A-to-C group was mediated in part by uncontrolled tubular cell proliferation triggered during the regeneration process after the ischemic insult. Although a previous study proposed that the increase in cellular proliferation is a necessary process to regenerate tubular epithelium in a period that typically is completed within 4 weeks after ischemia,[47] recent evidence demonstrated that ischemic, nephrotoxic, and obstructive injuries to the kidney induce a G2/M cell cycle arrest in the proximal tubular epithelial cells, sustaining proliferation indefinitely.[48] In this regard, Wynn[49] has proposed that this cell cycle arrest converts normal epithelial cells to a phenotype that promotes the growth and activation of fibroblasts, turning-on the fibrotic process after an ischemic insult. In addition, TGF-β has been recognized as a key mediator of the genesis of renal fibrosis.[32,50] TGF-β also contributes to the fibrogenesis through inducing epigenetic modifications in fibroblasts in a process that includes the hypermethylation of the gene encoding RASAL1 by the methyltransferase Dnmt1.[51] Accordingly, we found that tubulointerstitial fibrosis developed in the A-to-C group and was associated with increased expression and activation of TGF-β, as evidenced by increases in phospho-Smad3 and its target ECM proteins. Interestingly, activation of the TGF-β signaling pathway was not observed in the animals treated with spironolactone before or after (high dose) ischemia. These data highlight the contribution of TGF-β in mediating renal fibrosis after an ischemic insult. Although Smad2 and Smad3 interact and mediate TGF-β signaling, one recent line of evidence has shown that phospho-Smad2 may act as an antifibrotic effector of the TGF-β pathway.[23] In agreement with those results, the renoprotection conferred by spironolactone was associated with increased levels of phospho-Smad2.

The epithelial-to-mesenchymal transition has been suggested to promote tubulointerstitial fibrosis.[52,53] Under pathological conditions, tubular cells may dedifferentiate into myofibroblasts. TGF-β appears to promote this process by activating the Smad, ILK, and ERK pathways, as observed in tubular cells.[53] To monitor epithelial-to-mesenchymal transition, α-SMA protein levels were measured in the kidney. As expected, the renal fibrosis observed in the A-to-C group was associated with increased α-SMA protein levels. CKD progression has also been linked to an imbalance in free-radical production and antioxidant defense.[44,54] We confirmed that the rats that developed CKD exhibited greater urinary H_2O_2 excretion and a reduction in catalase activity. These changes in α-SMA levels and antioxidant factors were not observed in the A-to-C + Sp group.

In summary, we characterized an experimental model of CKD induced by AKI in the rat. Several mechanisms were responsible for CKD development, including increased

tubular cell proliferation, increased TGF-β pathway activation and oxidative stress, and overexpression of proinflammatory cytokines. By using this model of CKD, we demonstrated that the prevention of AKI with spironolactone completely prevents the progression to CKD. Moreover, we also demonstrate that administering an MR blocker after the ischemic insult also prevented CKD. Our results suggest that the treatment of patients with spironolactone after an AKI episode could be of help in preventing the development of CKD.

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 the Animal Care and Use Committee at our Institution.

Protocol 1

In all, 62 male Wistar (weighing 270–300 g) rats were divided into four groups: (1) rats subjected to sham surgery, $n = 9$ (S); (2) rats treated with spironolactone at 20 mg/kg per day by gastric gavage 3 days before sham surgery, $n = 9$ (Sp); (3) rats undergoing bilateral ischemia for 45 min, $n = 28$ (A-to-C); and (4) rats that received spironolactone 3 days before bilateral ischemia, $n = 13$ (A-to-C + Sp). All animals were observed for 9 months. In addition, four rats from the S, A-to-C, and A-to-C + Sp groups were included and observed for 10 days. All animals were kept in a 12:12 h day-night cycle and with free access to water and food.

Protocol 2

Because we recently demonstrated that postischemia MR blockade is beneficial in the prevention of AKI, with the best renoprotection observed in the first 3 h after ischemia,[22] this set of experiments was designed to evaluate whether postischemia spironolactone administration confers protection against the development of CKD. In all, 33 male Wistar (270–315 g) rats were divided into seven groups: sham-operated rats (S); rats subjected to bilateral renal ischemia for 45 min (A-C); and five groups of rats that underwent bilateral renal ischemia for 45 min, but also received one dose of spironolactone at 20 mg/kg by gastric gavage at 0, 1.5, or 3 h after ischemia (A-to-C, 0 h; A-to-C, 1.5 h; and A-to-C, 3 h, respectively), or that received a higher dose of spironolactone (80 mg/kg) at 0 or 1.5 h after ischemia (A-to-C 80, 0 h and A-to-C 80, 1.5 h). These animals were followed up for 3 months.

All other methods are described in the Supplementary Materials online.

Statistical analysis

The results are presented as the mean ± s.e. The significance of the differences between the groups was assessed by analysis of variance (ANOVA) using the Bonferroni correction for multiple comparisons. All of the comparisons passed the normality test. The differences in the ranks of glomerular diameters among the groups were evaluated by contingency analysis, and the differences were assessed using the χ^2 test with the Yates correction. Statistical significance was defined as P-value < 0.05.

DISCLOSURE

All the authors declared no competing interests.

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Disclaimer

The results presented in this paper have not been published previously in whole or in part, except as an abstract presented at the Annual Meeting and Scientific Exposition 2011 of the American Society of Nephrology (Philadelphia, PA).

SUPPLEMENTARY MATERIAL

Figure S1. Renal injury observed 90 days after inducing ischemia.
Figure S2. Glomerular and tubular injury is reduced by a low dose of spironolactone administrated after ischemia.
Figure S3. Glomerular and tubular injury is prevented by a high dose of spironolactone administrated after ischemia.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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Review Article

Epigenetic regulation in the acute kidney injury to chronic kidney disease transition

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SUMMARY AT A GLANCE

A fairly comprehensive review focusing on acute kidney injury and epigenetic regulators.

ABSTRACT:

Epigenetic modifications have emerged as a new, important contributor to gene expression regulation in both normal and pathophysiological conditions. Epigenetics have been studied in many diseases and conditions such as acute kidney injury (AKI), a syndrome with a high prevalence that carries a poor prognosis with increased morbidity and mortality. In addition, it has recently been shown that AKI increases the risk for the development of chronic kidney disease (CKD). The specific molecular mechanisms by which AKI increases the risk of CKD and end stage renal disease (ESRD) remain unknown, although there is new evidence supporting a role of epigenetic changes. The most studied epigenetic regulations in AKI are chromatin compaction, DNA methylation, and histone acetylation/deacetylation. These modifications predominantly increase the production of pro-inflammatory and profibrotic cytokines such as: monocyte chemoattractant protein-1 (MCP-1), complement protein 3 (C3), transforming growth factor β (TGF-β) that have been shown for perpetuating inflammation, promoting epithelial-to-mesenchymal transition (EMT) and ultimately causing renal fibrosis. A review of epigenetic mechanisms, the pathophysiology of AKI and recent studies that implicate epigenetic modifications in AKI and in the transition to CKD are discussed below.

EPIGENETICS

In the early 1940s, Conrad Hal Waddington used the term epigenetic, epi (above) and genetics (genetic), for the first time.[1] An epigenetic modification is defined as any potentially stable and, ideally, heritable change in gene expression or cellular phenotype that occurs without changes in Watson-Crick base-pairing of DNA.[2] The most described epigenetic regulations included chromatin compaction, DNA methylation, histone acetylation/deacetylation, and the noncoding sequences of RNA. Methylation could occur at DNA or at histones. In DNA, a methyl group is added to the cytosine or adenine nucleotides, whereas, in histones, a methyl group is transferred to lysine or arginine of histone proteins. Methylation can occur in different ways: mono-, bi-, or trimethylation in lysine residues and mono- or dimethylation in arginine residues. In contrast, acetylation only occurs in the lysine residues of histones, in which an N-terminal tail protruding is acetylated. The modifications in the amino tail of histones H3 and H4 play a crucial role in gene expression regulation. There are at least eight types of histone post-translational modifications: methylation on lysine or arginine, acetylation and deacetylation of lysines, phosphorylation on serine or threonine residues, ubiquitylation and sumoylation of lysines, and there are more than 60 different residues in which these modifications have been detected. Other mechanisms such as the small, mitochondrial or long non-coding RNA, are implicated. The precise moment when these post-transductional modifications occur depends on cell conditions and they never happen at the same time or in the same histone.

Complex organisms must condense their DNA using proteins that form a complex called chromatin. Chromatin is mainly composed by histones, which condense or relax the DNA in order to form two types: the heterochromatin (compact-chromatin) and the euchromatin (relaxed-chromatin).[3] The functional and basic unit is the nucleosome, which is formed by an octamer of histones. There are different histones: H2A, H2B, H3, H4 and H1. A pair of each of the former four comprises an octamer in

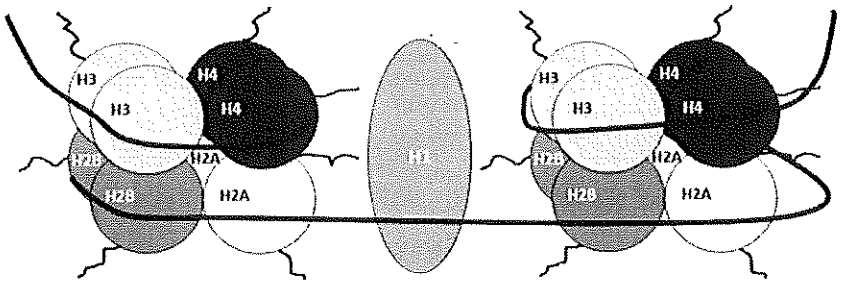

Fig. 1 Histones and DNA pearl necklace arrangement. Chromatin structure is formed by sets of nucleosomes. Each nucleosome contains a pair of histones: H2A, H2B, H3, and H4, which conform the functional octamer. Around the nucleosomes, DNA is wrapped two times. The H1 protein contains DNA from 10 to 80 bp of length that connects one nucleosome with the other one.

which DNA is wrapped two times. A DNA linker chain, connects the nucleosomes. This structural arrangement is condensed 10 times more than the relaxed DNA. The histone H1 functions as a 'linker' protein and assists in the formation of a chromatin fibre, which is formed by a tubular arrangement of nucleosomes and condensed 50 times compared to the relaxed DNA[3,4] (Fig. 1).

The DNA methylation seems to be the most understood method of epigenetic regulation. This occurs in the cytosines of cytosine-guanine (CpG) sites. DNA sections rich in CpG sites are associated with 76% of the promoter regions in the genome. The enzymes responsible for the methylation of DNA are called methyl-transferases (Dnmt). Dnmt1 and Dnmt2 are considered as maintenance methyltransferases, whereas Dnmt3 acts as a 'novo' methyl-transferase. Dnmt1 and Dnmt2 are responsible for attaching methyl groups to DNA hemi-methylated portions during replication, while Dnmt3 acts after replication.[5]

The histones acetylation state is regulated by the activity of histone acetylases (HAT) and deacetylases (HDAC). Normally, the acetylation of H3 and H4 increases the expression of genes involved in the open structure of the chromatin.[6]

Influence of the environment in epigenetic patterns

Manuel Esteller *et al.* demonstrated that monozygotic twins who were exposed to different environmental conditions during their lives had different patterns of methylation and acetylation within their genome affecting the gene expression of each twin. Conversely, when twins grow under the same conditions, patterns of methylation and acetylation are almost identical.[7] Additional evidence is found in studies of bees, where it has been shown that the larvae that are bound to be future queens are separated from the rest and fed a special mixture of honey, commonly called royal jelly, which has a high content of HADC10. This diet changes the acetylation pattern and phenotypic characteristics typical of a queen bee.[8]

RNA as an epigenetic regulator

In the last decades, the noncoding sequences of RNA (ncRNA) have been proven to play a key role in epigenetic regulation. It is now known that they serve a wide range of functions including the facilitation of chromosome dynamics, alternative splicing, transcriptional inhibition and destruction of mRNA. Despite the vast number of noncoding RNAs, the ones considered to be the most important in epigenetic silencing include: siRNAs, which are small molecules of 21–25 nucleotides, miRNAs, which are also small molecules that originate as endogenous precursor helix loop structures, and lncRNAs, which are bigger molecules with a length of 17 kilobases. The siRNAs and miRNAs regulate gene expression by an RNA interference phenomenon causing a repression at the translational level, while the lncRNAs work by modulating chromatin states and consequently modulating gene expression.[9]

ACUTE KIDNEY INJURY

Acute kidney injury (AKI) is a syndrome characterized by a sudden decline in renal excretory function, with a consequent failure in maintaining fluid, electrolyte and acid-base balance. AKI is diagnosed by clinical and laboratory manifestations such as oliguria (not always present) and elevations in serum creatinine, urea, phosphate and potassium concentrations.[10]

This syndrome was first described in the 1940s in London, where a report of cases of renal failure related to crush injuries was published.[11] In 2004, the Acute Dialysis Quality Initiative group published the Risk, Injury, Failure, Loss, and End-stage renal disease (RIFLE) classification in which the term 'acute kidney injury' and a formal definition were established. This definition evolved further into the AKI Network (AKIN) and in Kidney Disease: Improving Global Outcomes (KDIGO) definition.[12]

KDIGO defined AKI as: an increase in serum creatinine (SCr) equal to or greater than 0.3 mg/dL (>26.5 μmol/L) within 48 h; an increase in SCr equal to or greater than 1.5 times baseline, which is known or presumed to have occurred within the prior 7 days; or a urine output volume less than 0.5 mL/kg per h for 6 h. It also classifies AKI into three different stages (summarized in Table 1).[13]

It is difficult to assess the prevalence of AKI in the community. Nevertheless, there are some epidemiological studies showing that the incidence of AKI is between 2000 to 4000 people per million-population/year.[14] AKI is more prevalent

Table 1 Acute kidney injury classifications

System	Serum creatinine criteria	Urine output criteria
RIFLE class		
Risk	Serum creatinine increase to 1.5-fold OR GFR decrease >25% from baseline	<0.5 mL/kg per h for 6 h
Injury	Serum creatinine increase to 2.0-fold OR GFR decrease >50% from baseline	<0.5 mL/kg per h for 12 h
Failure	Serum creatinine increase to 3.0-fold OR GFR decrease >75% from baseline OR serum creatinine ≥354 μmol/L (≥4 mg/dL) with an acute increase of at least 44 μmol/L (0.5 mg/dL)	Anuria for 12 h
AKIN stage		
1	Serum creatinine increase ≥26.5 μmol/L (≥0.3 mg/dL) OR increase to 1.5–2.0-fold from baseline	<0.5 mL/kg per h for 6 h
2	Serum creatinine increase >2.0–3.0-fold from baseline	<0.5 mL/kg per h for 12 h
3	Serum creatinine increase >3.0-fold from baseline OR serum creatinine ≥354 μmol/L (≥4.0 mg/dL) with an acute increase of at least 44 μmol/L (0.5 mg/dL) OR need for RRT	<0.3 mL/kg per h for 24 h OR anuria for 12 h OR need for RRT

GFR, glomerular filtration rate; RRT, renal replacement therapy.

in the inpatient population, 3.2% to 9.6% of admissions[15] and is especially common in critically ill patients, 40% to 60%.[16]

The diagnosis and the identification between the different causes of AKI can be challenging given the limited sensitivity and specificity of biochemical parameters. But, significant progress has been made in the identification of diverse biomarkers like neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (Kim-1) interleukin-18 (IL-18), fatty acid-binding protein 1 (L-FABP1), and heat shock protein 72 (Hsp72), although their widespread use has been limited for different reasons (for review[17]).

There are a myriad of causes of AKI. For didactic and clinical reasons, they are typically divided into pre-renal (hypo-perfusion of the kidney), intrinsic renal (pathologic process within the kidneys), and post-renal causes (obstruction of urine flow distal to the kidneys), although AKI is often multifactorial. It is believed that pre-renal and post-renal AKI begin as functional processes, and if they are corrected in a timely manner, any residual renal damage can be limited. Intrinsic AKI, however, represents structural damage.[18] But, many authors consider AKI to be a continuum of injury that starts with pre-renal AKI.[19]

The cellular and molecular events that take place during an event of AKI can be divided into four phases: (i) 'The initiation' phase, which takes place as a direct consequence of decreased renal blood flow (RBF) resulting in significant cellular ATP depletion and tubular epithelial cell injury with typical histologic alterations (loss or inversion of polarity and loss of adhesion to the basement membrane).[20] In this phase there are releases of cytokines and chemokines that initiate the inflammation cascade. (ii) Sustained hypoxia due to vascular endothelial cell damage and an intense inflammatory response characterizes 'the extension' phase, which is marked by renal epithelial cell injury and death by both necrosis and apoptosis. Some have argued that it is during this phase that a therapeutic intervention could be most successful by preventing the amplification of inflammation.[21] (iii) 'The maintenance' phase, where the inflammation becomes regulated and there is an attempt to re-establish and maintain cellular and tubular integrity. There is marked epithelial cell repair, migration and proliferation, and (iv) 'The recovery' phase in which cellular differentiation continues. After recuperation of the renal epithelial cell polarity, normal organ function returns.[22]

Despite the progress in the understanding of the pathophysiology of AKI, the cornerstone of treatment remains supportive care. There are some medications that offer a theoretical benefit such as loop diuretics or low doses of dopamine. However, these drugs have not shown any significant improvement in renal outcomes in different meta-analyses.[23] There is some evidence demonstrating that receptor mineralocorticoid antagonism,[24–27] anti-inflammatory,[28] and antioxidant therapies[29] could improve outcomes in AKI, although large clinical trials are lacking.

AKI TO CKD TRANSITION

In the past, the renal outcomes in patients after recovery from AKI were believed to be benign with a low probability of developing chronic kidney disease (CKD) and end-stage renal disease (ESRD). Nevertheless, in the last few years, evidence has revealed a strong association between AKI and the consequent development of CKD. Most of the evidence comes from large observational studies that have consistently shown that a significant number of patients with AKI, even those patients without prior kidney disease, after partial or complete recovery of renal function often then progress to advanced stages of CKD or even ESRD in some cases. The number of episodes, the severity of AKI and pre-existing kidney disease appear to correlate with a greater risk for progression to CKD.[30] In this regard, Bucoloiu ID *et al.*[31] showed that 6.6% of AKI patients that had a complete renal function recovery exhibited a greater risk of de novo CKD and death in the following months. Furthermore, a meta-analysis that included 13 large studies found that AKI is an independent risk factor for CKD.[30] Worldwide, it has been shown that 20% of patients with an AKI episode will develop CKD after 3 years.[32]

As was commented before, during AKI a number of processes are activated to repair the affected renal structures, but they can lead to cell proliferation, hypertrophy and exaggerated extracellular matrix production.[33] Renal vasoconstriction predominates after AKI due to an increase of endothelin-1, angiotensin II, thromboxane A2, and adenosine, as well as, by the reduction in nitric oxide (NO) synthesis.[34–37] These effects are more enhanced by increased leukocyte adhesion to the endothelium, which occludes small vessels and compromise renal vascular microcirculation.[38] In addition, the number of renal vessels decreases as a result of capillary rarefaction phenomenon.[39–41] This process seems to be facilitated by the vascular endothelial growth factor reduction.[42,43] As a result, chronic hypoxia leads to progressive deterioration of the tubular epithelium, leading to cell cycle arrest and epigenetic alterations that eventually cause the progressive development of tubulointerstitial fibrosis.[44,45]

IS EPIGENETIC REGULATION A NEW FACTOR IN THE TRANSITION FROM AKI TO CKD?

It is not surprising to find that epigenetic regulation may participate in AKI to CKD transition because of its implication in the cell adaptation to extreme circumstances, such as oxidative stress, hypoxia, and mitochondrial injury.[46]

Chromatin structure modifications

During an AKI episode, the tubular epithelial cells are subjected to a hypoxic milieu, causing modification not only in the cellular metabolism, but also in the chromatin structure and in the binding of different transcription factors.[47] It is well known that there is an increase in the expression of pro-inflammatory cytokines such as tumour necrosis factor (TNF-α) and monocyte chemoattractant protein (MCP-1) after an AKI episode, which persists until 7 days.[48,49] This effect seems to be the result of epigenetic regulation, because there is an increment in the multiprotein chromatin remodelling complex that includes the SWItch/Sucrose Non-Fermentable (SWI/SNF) factor. This complex depends on helicase-like ATPase activity and regulates chromatin structure. The ATPases of this complex are the machinery that allow dynamic changes in chromatin structure by activating or inactivating gene expression. Specifically, the human SWI/SNF complex is also able to slide nucleosomes along the DNA, promoting the transcription start sites and making them more accessible for specific genes. This complex contains the Brahma-related gene1 (BRG1), which is an ATPase catalytic chromatin remodelling subunit. In the mice, BRG1 is a regulator of the nucleosome remodelling complexes in the TNF-α gene.[49] Recent findings have shown that there is also an increase in MCP-1 independent of the causes of AKI[47] (Fig. 2A).

Histone epigenetic modifications during AKI

Adequate cholesterol synthesis helps to preserve the epithelial cells during an ischaemic insult because cholesterol regulates both plasma membrane integrity and mitochondrial function. Naito M *et al.*[50] found that after 3-days of ischaemia, there was an increase in the RNA polymerase II recruitment (Pol II) and sterol regulatory element binding proteins-1 and 2 (SREBP-1 and SREBP-2) to co-enzyme A reductase (HMGCR), which results in enhanced cholesterol synthesis – this recruitment is possible because, after renal ischaemia/reperfusion (I/R), the trimethylation of histone 3-lysine 4 (H3K4m3) and the acetylation of histone 3-lysine 9 (H3K9) occur. These coordinated events led to greater epithelial cell survival during an ischaemic event (Fig. 2B).

ATF3 belongs to the activating transcription factor/cAMP responsive element-binding protein (ATF/CREB) family and has been identified as a transcriptional repressor. During I/R injury there is an induction of ATF3 in the kidney. Accordingly, ATF3-deficient mice exhibited greater renal I/R-induced mortality, kidney dysfunction, inflammation and proximal tubular apoptosis compared with wild-type mice. When ATF3 was re-established in the kidney, rescue of the renal I/R-induced injury was observed, suggesting that this factor increases the expression of cytoprotective molecules.[51] In addition, it has been proven that ATF3 interacts with histone deacetylase-1 (HDAC1) after I/R injury, which results in the condensation of chromatin, the interference of nuclear factor-κB (NF-κB) binding to the DNA, and the inhibition of inflammatory gene transcription such as, IL-2b and P-selectin (Fig. 2B). Unfortunately, the increase in cholesterol synthesis and ATF3 activation are not enough to prevent renal injury, but both are important mechanisms triggered by epithelial cells to reduce renal injury.

DNA epigenetic modifications during AKI

Another epigenetic modification is DNA methylation/demethylation. Pratt JR *et al.*[52] show the upregulation of complement C3 that was due to an increase in the demethylation of a cytosine residue in the interferon-γ (IFN-γ) responsive element within the C3 promoter in the rat kidney that had undergone I/R. Accordingly, Huang N *et al.*[53] confirmed these findings in C57BL/6 mice that underwent renal I/R. Moreover, they demonstrated that this epigenetic modification promoted a decrease in Tet1 and Tet2, which catalyzes the oxidation of 5-methylcytosine (5-mC) into 5-hydroxymethylcytosine. This reduced oxidation was associated with an increase in the expression of IL-10 and IFN-γ receptor-2. Another interesting finding of this study was that the demethylation of the C3 promoter persisted for at least 6 months in transplanted rat kidneys (Fig. 2B). In a recent study, DNA methylation was evaluated in two groups of patients with CKD: with rapid decline in kidney function and with stable kidney function. Interestingly, the stable kidney function group exhibited a greater hypemethylation in

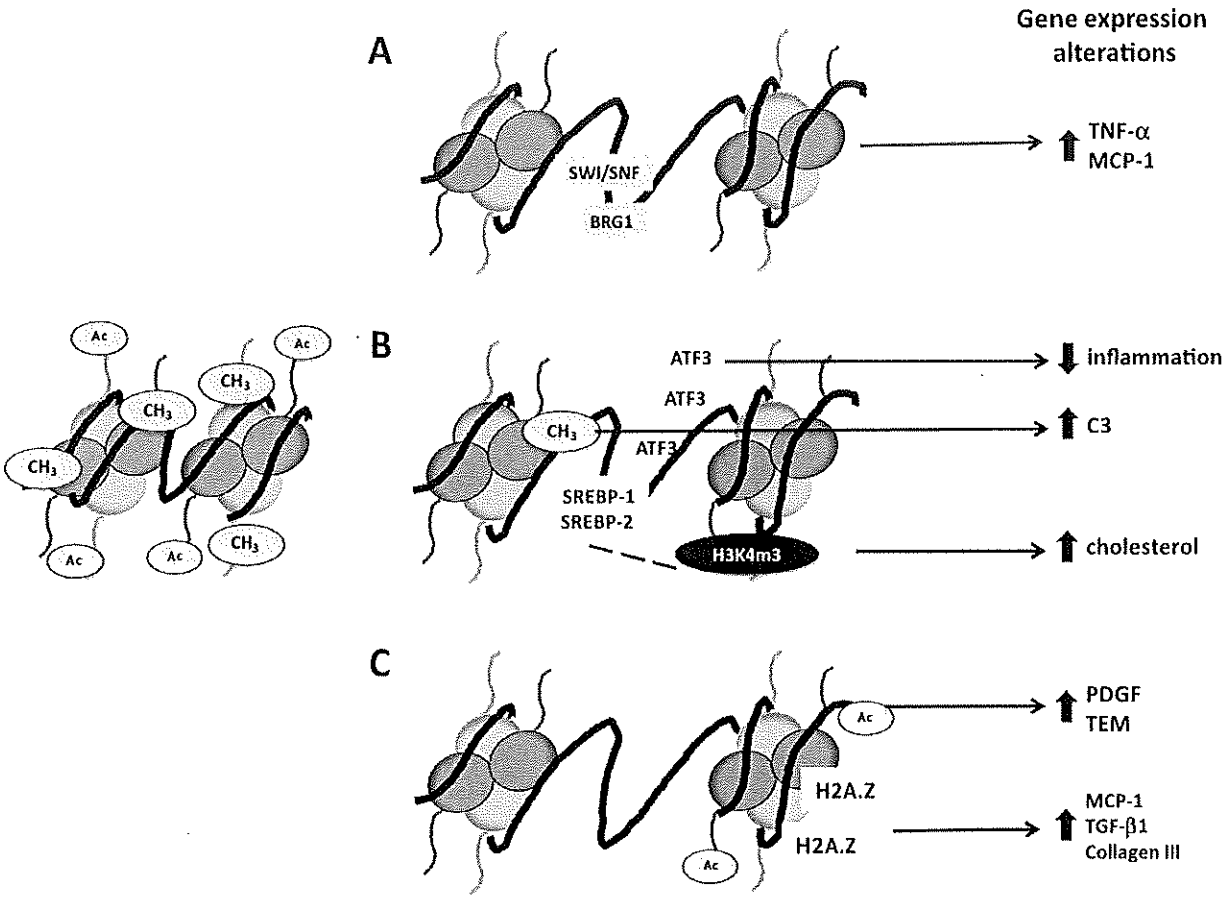

Fig. 2 Epigenetic modifications that occur after an episode of acute kidney injury. (A) Relaxation of chromatine which is mediated by SWItch/Sucrose Non Fermentable (SWI/SNF) factor, sliding the nucleosomes along the DNA and promoting the transcription start sites more accessible for specific genes. This complex contains the Brahma-related gene 1 (BRG1), a regulator of the nucleosome remodelling complexes in tumour necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) genes. (B) The DNA demethylation allows the recruitment of RNA polymerase II (Pol II) and sterol regulatory element binding proteins 1 and 2 (SREBP-1 and SREBP-2) to co-enzyme A reductase (HMGCR), which results in enhanced cholesterol synthesis that leads to a greater survival of the epithelial cell during an ischaemic event. Also, there is an induction of the transcriptional repressor ATF3 in the kidney, which helps to reduce the kidney dysfunction, inflammation and proximal tubular apoptosis. In addition, complement C3 upregulation is observed as a result of an increase in demethylation of a cytosine residue in the interferon-γ responsive element within the C3 promoter. This demethylation of the C3 promoter has been reported to persist at least 6-months in transplanted rat kidneys. (C) Histones deacetylation promotes myofibroblast proliferation and epithelial-to-mesenchymal transition (EMT). In addition, the increased expression of histone 2 variant (H2A.Z) promotes the increase n of fibrotic and inflammatory genes such as MCP-1, transforming growth factor-β (TGF-β1), collagen III.

nephronophthisis 4 (NPHP4), IQ motif, Sec7 domain (IQSEC1), GEP100, and transcription factor 3 (TCF3), genes involved in the epithelial-to-mesenchymal transition (EMT), suggesting that this epigenetic modification confers certain renoprotection in these patients with lower rate of renal progression.[54]

Histone changes during AKI

The inhibition of HDAC activity by trichostatin A (TSA) decreased the proliferation induced by platelet-derived growth factor (PDGF) of NIH-3T3 skin fibroblasts. In addition, Pang M *et al.* used an in vivo model of unilateral urethral obstruction to demonstrate that TSA blocks the EMT induced by transforming growth factor-β (TGF-β) in renal tubular epithelial cells, suppresses the expression of α-smooth muscle actin (α-SMA) and fibronectin and attenuates the accumulation of renal interstitial fibroblasts in the kidney (Fig. 2C).[55] Additionally, it has been reported that pharmacological HDAC inhibition promotes anti-inflammatory and antifibrotic effects in other diseases such as Alzheimer's, Parkinson's and multiple sclerosis.[56]

In 2008, Marumo T *et al.*[57] reported a transient decrease in histone acetylation in the proximal tubular cells of mice that

was appreciated immediately following severe unilateral I/R. This effect was recovered after 24 h due to a decrease in HDAC5. In a second study, these authors reproduced these findings and showed that HDAC5 knockdown by RNAi significantly increased histone acetylation and upregulated BMP7 expression promoting the tubular epithelium recovery.[58] In contrast, Zager *et al.*[59] used an enzyme-linked immune-absorbent assay (ELISA) of renal cortexes and found an increase in renal acetylated histone H3 levels after I/R; these changes were seen 24 h after injury and persisted for 3 weeks. This discrepancy could be explained through a different temporal response following I/R.

Although histone acetylation modifications appear to be involved in the transition of AKI to CKD, the story is more complicated, because it has been demonstrated in mice that underwent unilateral I/R that two gene-activating histone alterations also occur: histone 3, lysine 4 trimethylation (H3K4m3) and increased expression of histone 2 variant (H2A.Z). Both changes promote an increase in the expression of fibrotic and inflammatory genes such as MCP-1, TGF-β1 and collagen III[48] (Fig. 2C). Moreover, Marumo T *et al.*[58] explored the role of HDAC in tubulointerstitial injury by using the model UUO, in which HDAC1 and HDAC2 are activated and are responsible for reducing histone acetylation in the injured kidney. As expected, TSA treatment attenuated macrophage infiltration and tubulointerstitial fibrosis. The induction of colony-stimulating factor-1 (CSF-1), a chemokine known to be involved in macrophage infiltration in tubulointerstitial injury, was also reduced. Accordingly, the knockdown of HDAC1 or HDAC2 significantly reduced CSF-1 induced by TNF-α in renal tubular cells.

Another epigenetic modification described in cancer and diabetes is the dysfunction of histone acetyl-transferases (HATs), which also have been seen as potential targets for the design of new therapies. In renal I/R injury, the administration of curcumin (diferuloylmethane), which is a specific inhibitor of HAT (p300/CREB-binding protein), reduced oxidative stress and improved renal function, suggesting the participation of HAT in promoting renal injury.[62]

After AKI, the surviving epithelial cells proliferate to re-establish the normal tubular structure, although some of these cells may remain arrested in the G2/M cell cycle phase, delaying renal structure recovery and promoting the development of chronic fibrosis.[44,45] Thus, it is reasonable to hypothesize that drugs able to stimulate the cell cycle may have a beneficial effect on this pathology. Accordingly, m4PTB, a histone deacetylase inhibitor, was able to promote renal progenitor cell proliferation, accelerate the recovery of AKI induced by gentamicin in zebrafish, and reduce renal ischaemic injury in mice. The protective effect of m4PTB was associated with increased proliferation of the tubular cells mediated by both inducing the expression of genes involved in the cell cycle and with a higher number of cells in the S-phase. Long-term kidney fibrosis was also reduced by m4PTB.[60] Similarly, Novitskaya T *et al.*[61] recently showed that the administration of phenylthiobutanoic acids (PTBAs), a new class of histone deacetylase (HDAC) inhibitor, was also able to accelerate the AKI recovery and reduce fibrosis in a progressive model of AKI induced by aristolochic acid, due to increased tubular proliferation and decreased G2/M cell cycle arrest. In addition, Richard A Zager *et al.*[59] showed in mice that the progressive renal disease observed throughout the 3 weeks after ischaemia was associated with a progressive increase from 5% (at baseline) to 75% (at 3 weeks) in pro-inflammatory cytokine/chemokine genes such as: MCP-1, TNF-α, and TGF-β1. These changes were in accord with a progressive gene-activating H3 acetylation.

All of these studies together suggest that epigenetic changes that occur after an ischaemic insult can persist despite the resolution of the AKI episode and seem to be partially responsible for the persistent inflammation, profibrotic milieu and EMT that have been shown to contribute to CKD development. Further research is still needed regarding these findings, but these are promising findings that provide opportunities to find and develop new targets to prevent AKI and the progression to CKD.

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DISCLOSURE

The authors declare no financial conflict of interest.

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Research Paper

Mild ischemic Injury Leads to Long-Term Alterations in the Kidney: Amelioration by Spironolactone Administration

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Abstract

Administration of the mineralocorticoid receptor antagonist spironolactone prevents the development of chronic kidney disease (CKD) after a severe ischemic injury. However, whether brief periods of ischemia lead to CKD and whether spironolactone administration after ischemia may be a useful therapeutic strategy to prevent the gradual deterioration of structure and function remains unexplored.

Nineteen male Wistar rats were divided into four groups: rats that underwent renal bilateral ischemia for 10, 20, or 45 min were compared with sham operated rats. Additionally, thirteen male Wistar rats that underwent renal bilateral ischemia for 20 min were divided into an untreated ischemic group (I) and two groups receiving spironolactone, 20 mg/kg by gavage, at either 0 (Sp0) or 1.5-h after ischemia (Sp1.5). The rats were followed up and studied after 9 months.

Mild (20 min) and severe (45 min) ischemia induced a progressive increase in proteinuria at varying magnitudes, whereas minor ischemia (10 min) did not modify proteinuria. CKD induced by moderate ischemia was characterized by renal hypertrophy and tubulointerstitial fibrosis. These effects were associated with activation of the transforming growth factor β (TGFβ) signaling pathway and up-regulation of endothelin receptor A (ETA) and alpha smooth muscle actin (αSMA). Spironolactone treatment immediately or 1.5-h after the ischemic insult prevented the onset of these disorders.

Our results show that moderate ischemic insult leads to long-term structural and molecular changes that may compromise renal function in later stages. Additionally, we demonstrate that spironolactone administration after mild ischemia prevents this detrimental effect.

Key words: Aldosterone, fibrosis, acute kidney injury, chronic kidney disease

Introduction

Ischemia/reperfusion injury is the main cause of acute kidney injury (AKI) occurring in approximately 15% of hospitalized patients, and its incidence rises to 40-60% in intensive care unit (ICU) patients (1) (2) (3). Despite recent advances in new therapies and the knowledge of the mechanisms involved in AKI, this syndrome has high morbidity and mortality rates (4).

More worrisome is the recent accumulating evidence indicating that patients who survive an AKI episode have a higher risk of developing chronic

kidney disease (CKD) in the following years (5). Therefore, AKI has been recognized as a risk factor for the development of CKD (6) (7). A recent study showed that 6.6% of AKI patients who had complete recovery of renal function had a greater risk of death and de novo CKD in the following months (8). A recent meta-analysis that included thirteen large studies found that AKI is an independent risk factor for CKD (9). Worldwide, 20% of patients with an AKI episode will develop CKD after 3 years, which represents 300,000 patients in high-income countries, and this value might be higher than 1.8 million in low- and middle-income countries (10).

In support of several epidemiological studies, animal models have shown that after a renal ischemia/reperfusion event, the recovery process may be incomplete, producing progressive renal dysfunction, tubulointerstitial fibrosis and chronic inflammation (for review (11)). We have previously shown that aldosterone plays a key role in the physiopathology of renal injury induced by ischemia. In this regard, we showed that adrenal gland removal or mineralocorticoid receptor (MR) blockade with spironolactone before or even after ischemia prevents the acute (24 h) functional and structural injury induced by I/R (12-14). Interestingly, CKD was prevented when spironolactone was administered upon severe ischemic injury, and the untreated ischemic group developed progressive renal dysfunction, proteinuria, glomerular hypertrophy, glomerulosclerosis, aberrant tubular dilation and tubule-interstitial fibrosis (15). These results suggest that MR blockade is a powerful strategy to prevent CKD induced by a longer period of ischemia in the rat (15). However, this severe ischemic injury model might only be applicable to patients undergoing cardiovascular surgery or renal transplantation.

The probability of developing CKD or end-stage renal disease (ESRD) over time is proportional to the severity and the duration of the AKI event (16). Until now, studies performed in rats have explored the effects of severe ischemic injury (45 to 60 min of renal ischemia) on long-term renal functional and structural deterioration (15;17-24). Because in most of the patients AKI occurs unexpectedly, and renal injury appears as a result of a lower degree of hypoperfusion, we addressed the following issues in this study: whether mild ischemic injury (20 min) is able to induce chronic renal injury, and whether spironolactone administration post-ischemia is effective in preventing the long-term effects of mild ischemia.

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 the Animal Care and Use Committees at our institutions (Comisión de Investigación en Animales del Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubiran and Comisión Institucional para el Cuidado y Uso de Animales del Laboratorio del Instituto de Investigaciones Biomédicas).

To test the impact of various durations of ischemia on the progressive increase of proteinuria, nineteen male Wistar rats (270-300 g) were divided into four groups: sham-operated (n=4), I/R of 10 min (n=5), I/R of 20 min (n=5), and I/R 45 min (n=5). To investigate the efficacy of MR antagonism on the long-term effects of mild ischemia, thirteen male Wistar (270-300 g) rats were divided into three groups: rats that were subjected to bilateral renal ischemia for 20 min (I, n=5) and two groups of rats that underwent bilateral renal ischemia for 20 min receiving only one dose of spironolactone (20 mg/kg by gastric gavage) either immediately or 1.5 h after ischemia (Sp0, n=4 and Sp1.5, n=4, respectively). These groups were compared with the sham-operated group used in the first set of experiments.

Ischemia/reperfusion model

Rats were anesthetized with an intra-peritoneal injection of sodium pentobarbital (30 mg/kg) and placed on a heating pad to maintain rat core body temperature at 37 °C. Renal pedicles were isolated and bilateral ischemia was induced by the collocation of a non-traumatic clamps during 10, 20 or 45 minutes. Ischemia was verified visually by change in kidney color. Reperfusion was achieved by release of the clips and confirmed by return of blood to the kidney. The incision was closed in two layers with 3-0 sutures. For sham surgery, anesthesia, laparotomy and renal pedicle dissection, without clamp collocation was performed. After the surgery the rats were allowed to recover and followed up for 270 days.

Functional parameters

Urinary protein excretion was determined every 30 days throughout the follow up in all studied groups using the urine collected over a 24-h period. At the end of the experimental periods (9 months), rats were anesthetized with sodium pentobarbital (30 mg/kg) and placed on a homoeothermic table. The left femoral artery was catheterized with polyethylene tubing (PE-50). The mean arterial pressure (MAP) was monitored with a pressure transducer (model p23 db, Gould) and recorded on a polygraph (Grass Instruments, Quincy, MA). An ultrasound transit-time flow probe was placed around the left artery and filled with ultrasonic coupling gel (HR Lubricating Jelly,

Carter-Wallace, New York, NY) to record the renal blood flow (RBF). Blood samples were taken at the end of the study. Urine and serum creatinine concentrations were measured with Quantichrom creatinine assay kit (DICT-500), and renal creatinine clearance was calculated by the standard formula C = (U X V)/P, where U is the concentration in urine, V is the urine flow rate, and P is the serum concentration. Urinary protein excretion was measured by the TCA turbidimetric method (25).

Light microscopy analysis

Histopathological analysis was performed in all rats after 9 months. The right kidney was removed and the cortex and medulla were isolated; then, the tissue was frozen in liquid nitrogen and stored at -80° C. The left kidney was perfused through the femoral catheter with a physiological solution. Following blanching of the kidney, the perfusate was replaced by freshly prepared 10% neutral-buffered formalin, and perfusion was continued until fixation was completed. After appropriate dehydration, renal tissue was embedded in paraffin, sectioned at 4 μm and stained with periodic acid-Schiff (PAS) reagent or Sirius red stains. The degree of tubulointerstitial fibrosis was evaluated by morphometry in Sirius red-stained preparations (magnification x400). Accordingly, five to eight subcortical fields per section were randomly selected in kidneys from the groups studied. Tubulointerstitial fibrosis consisted of extra cellular matrix expansion with collagen deposition together with distortion and collapse of the tubules; fibrosis was evidenced by red coloration in Sirius red stained slides. The affected area was delimited, and the percentage of tubulointerstitial fibrosis was calculated by dividing the fibrotic area by the total field area, excluding the glomerular and tubular luminal areas. All of the slides were blindly analyzed.

Western Blot analysis

Total renal proteins were isolated from renal cortex from each rat and homogenized in lysis buffer (50 mM HEPES ph 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, and complete protease inhibitor (Roche). Protein samples containing 50 μg of total protein were resolved by 8.5% SDS-PAGE electrophoresis and electroblotted onto polyvinylidinedifluoride membranes (Millipore). Membranes were then blocked with 5 % blotting-grade non-fat dry milk. After that membranes were incubated in 0.1 % blotting-grade non-fat dry milk with their respective antibodies. Specific antibodies against α-smooth muscle actin (Sigma A2547, 1:5000), Smad3 (sc-101154, 1:500), phosho-Smad3 (Millipore, 1:500), TGF-β (sc-146, 1:500), ET_A (Abcam, 1:5000) and ET_B (Abcam, 1:5000) were used. After incubation with primary antibody, membranes were washed and incubated with their respective secondary antibody. As a loading control, membranes were incubated overnight at 4 °C with goat anti-actin antibody (Santa Cruz Biotechnology, 1:5000 dilution). Actin was detected using donkey anti-goat IgG-HRP (1:5000, Santa Cruz Biotechnology). Proteins were detected with an enhanced chemiluminescence kit (Millipore) and autoradiography, following the manufacturer's recommendations. The bands were scanned for densitometric analysis.

Endothelin ELISA

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

The results are presented as the mean ± S.E. The significance of the differences between the groups was assessed by analysis of variance (ANOVA) using the Bonferroni correction for multiple comparisons. All of the comparisons passed the normality test. Statistical significance was defined as having p values <0.05.

Results

Mild and severe ischemic insult leads to progressive increase in proteinuria.

As expected, 45 min of renal ischemia induced a progressive increase in proteinuria from 21.8 ± 3.4 (one month) to 320.4 ± 22.8 mg/24 h (nine months post-ischemia) (Figure 1). A brief period of ischemia (20 min) also induced a progressive increase in urinary protein excretion, although the extent was significantly lesser than in the group with 45 min ischemia, from 12.1 ± 2.2 (one month) to 189.6 ± 36.2 mg/24 h (9 months post-ischemia). In contrast, rats that suffered a minor ischemic insult (10 min) did not develop proteinuria after the nine-month follow-up.

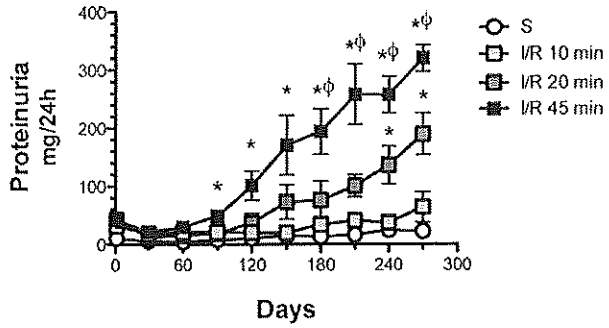

Figure 1. Effect of various durations of ischemia on proteinuria development. Four groups were included: sham (S), and rats that underwent bilateral renal ischemia of 10, 20 or 45 min duration. The urinary protein excretion was determined every 30 days: sham (white circles, n=4), I/R 45 min (black squares, n=5), I/R 20 min (dark gray squares, n=5) and I/R 10 min (gray squares, n=5). *p<0.05 vs. Sham-operated rats and ϕ p<0.05 vs. I/R 45 group.

The progressive increase in proteinuria induced by a brief ischemic period is reduced by spironolactone administration after the ischemic insult.

We next evaluated the effectiveness of post-ischemic treatment with spironolactone to prevent long-term functional, structural and molecular damage. The progressive increase of proteinuria induced by 20-min of ischemia was significantly lessened after nine months in the groups treated with spironolactone immediately (Sp0) or 1.5-h (Sp1.5) after ischemia (63.7 ± 18.5 and 66.1 ± 19.2 mg/day, respectively) (Figure 2A). Despite the presence of proteinuria in the untreated ischemic group, the rats did not exhibit renal dysfunction at the end of the experiment; similar values of renal blood flow and creatinine clearance were observed among the groups (Figures 2B and 2C). None of the rats presented with an increase in mean arterial pressure, indicating that phenotypic changes are directly related to the duration of ischemia and not secondary to systemic hypertension (Figure 2D).

Long-term renal structural changes induced by a brief ischemic period

Representative light microscopy sections from rat kidneys stained with periodic acid-Schiff are shown in Figure 3A-D. IR induced structural changes characterized by glomerular hypertrophy, glomerulosclerosis, and cast formation (Figure 3B). In contrast, the Sp0 and Sp1.5 groups exhibited glomerular and tubular architecture similar to those observed in sham-operated rats (Figure 3C-D). Accordingly, the untreated ischemic group exhibited an increase in the percentage of glomerulosclerosis (14.4%), which was not observed in rats treated with spironolactone (Figure 3E). Renal hypertrophy was evaluated by kidney weight. Despite similar body weights among the groups, the kidney weight and body weight ratio (KW/BW) was 40% higher in the IR group than in the sham operated group (0.0042 ± 0.0004 vs. 0.00291 ± 0.0001, p=0.03), as shown in Figure 3F. This renal hypertrophy was not observed in any of spironolactone-treated groups (0.0030 ± 0.0001 and 0.0032 ± 0.0001 for Sp0 and Sp1.5, respectively).

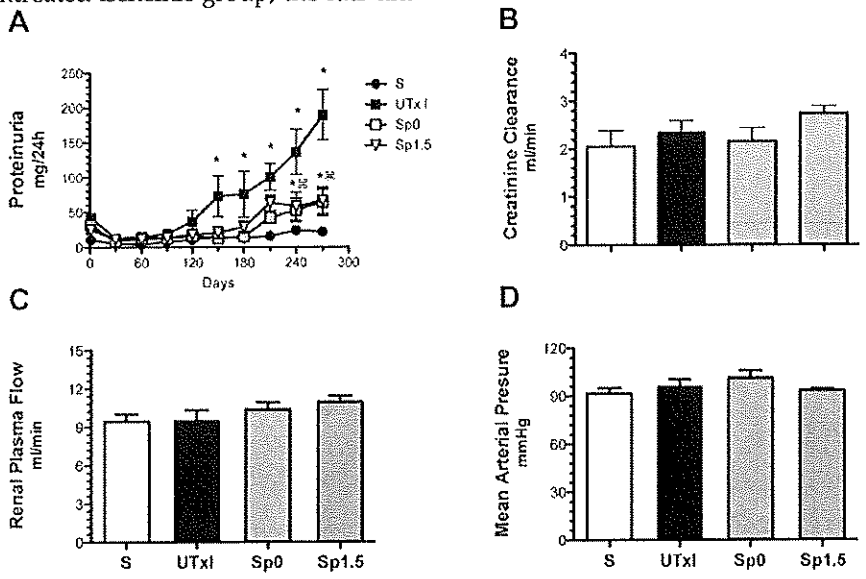

Figure 2. Mild acute kidney injury leads to the development of proteinuria, and the effect was ameliorated by spironolactone administration. Four groups were included: sham (S, n=4), rats that underwent bilateral renal ischemia for 20 min (UTxI, n=5) and rats that received spironolactone (20 mg/kg) at 0 or 1.5 hours after ischemia (Sp0, and Sp1.5, respectively, n=4). A) Urinary protein excretion was determined every 30 days during the follow-up: sham (black circles), A-C (black squares), Sp0 (gray squares) and Sp1.5 (gray triangles). At the end of the 9-month period, B) creatinine clearance, C) renal blood flow and D) mean arterial pressure were determined in the sham (white bars), untreated ischemic group (black bars), and spironolactone-treated groups (gray bars). *p<0.05 vs. sham operated rats and ⌘P<0.05 vs. the UTxI group.

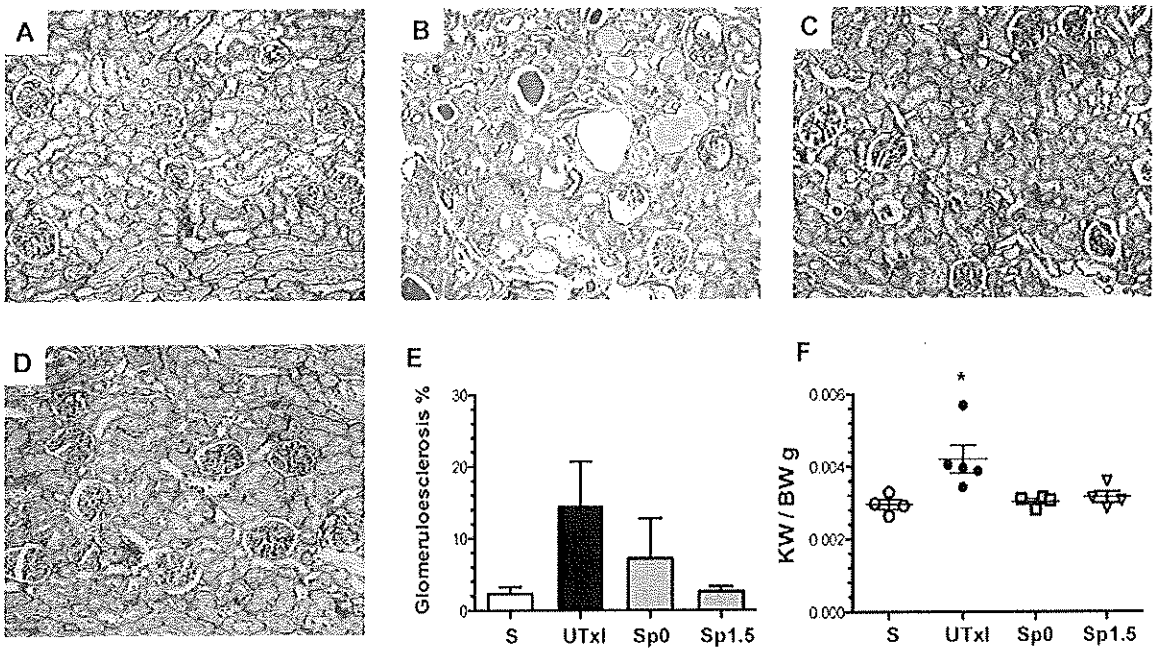

Figure 3. Twenty minutes of bilateral renal I/R led to renal structural injury, which can be prevented by spironolactone administration. Representative images of periodic acid–Schiff (PAS)-stained sections from A) Sham (n=4), B) untreated ischemic group (n=5), C) Sp0 (n=4) and D) Sp1.5 (n=4) groups. The main effects observed were: Tubular dilation, tubular cast formation and glomerular sclerosis. Original magnification: X100. E) Glomerulosclerosis percentage and F) Ratio between kidney weight and body weight (KW/BW). *p<0,05 vs. all the groups.

Figure 4 shows the representative microphotographs from kidney slides stained with Sirius red and the morphometric analysis of the various groups. The untreated ischemic group exhibited a significant area affected by tubulointerstitial fibrosis (Figure 4C-4D). In contrast, the spironolactone-treated groups showed practically no staining for Sirius red (Figure 4E-4H). These observations were confirmed by the morphometric analysis presented in Figure 4B.

Long-term molecular changes induced by a brief ischemic period and prevention by spironolactone.

The role of the TGF-β pathway in promoting the observed fibrosis was assessed. The ischemic untreated group exhibited a significant increase in TGF-β protein levels (Figure 5A). To assess the activation of this pathway, the renal levels of a downstream effector of the TGF-β pathway, phospo-Smad-3, were evaluated by Western blot analysis (Figure 5B). Accordingly, phospho-Smad3 was significantly elevated in the untreated ischemic group. Similar to the effect observed at the TGF-β level, the increased phosphorylation of Smad-3 was not observed in the spironolactone-treated groups. Additionally, the structural injury was associated with a significant up-regulation of renal α-SMA protein levels. This progressive increase was prevented in the spironolactone-treated groups (Figure 5C).

Recently, it was suggested that endothelin-1 activation and enhanced ET_A expression may contribute to the progression of AKI to CKD (18). For this reason, the protein levels of ET-1 and its receptors ET_A and ET_B were measured. Although no difference in the intra-renal content of ET-1 was observed (Figure 6A), we found an up-regulation of ET_A receptors in the untreated ischemic group, and the effect was prevented by spironolactone treatment (Figure 6B). Regarding the ET_B receptor, an up-regulation in all ischemic groups was observed (Figure 6C).

Discussion

The development of CKD in the years following an AKI episode is a major public health issue, and it is associated with poor quality of life in the patients and increased expenses to the health system. The association between AKI and CKD has been consistently recognized in several epidemiological studies. For example, complete recovery of renal function after an episode of AKI in patients with normal baseline kidney function is associated with increased risk of the development of incident stage 3 CKD (5). Moreover, Chawla LS et al. showed that the severity of the AKI episode is strongly associated with the risk of CKD progression (16). However, most of the experimental studies, including a study from our laboratory (15;19;26-29), investigating the possible association between AKI and CKD have focused on the long-term effects of a severe ischemic lesion; therefore, more

information on the effects of a brief period of ischemia on chronic renal injury is required. In this study, we first characterized whether the severity of the ischemic insult was associated with the presence and intensity of chronic renal injury induced by renal bilateral ischemia. Rats that underwent a longer period of ischemia (45 min) developed heavy proteinuria beginning in the third month after ischemia, as we previously reported (15). A mild period of ischemia (20-min) also induced a progressive increase in proteinuria, but this was evident only after 5 months of the initial insult and was less severe compared with the longer-period ischemia group.

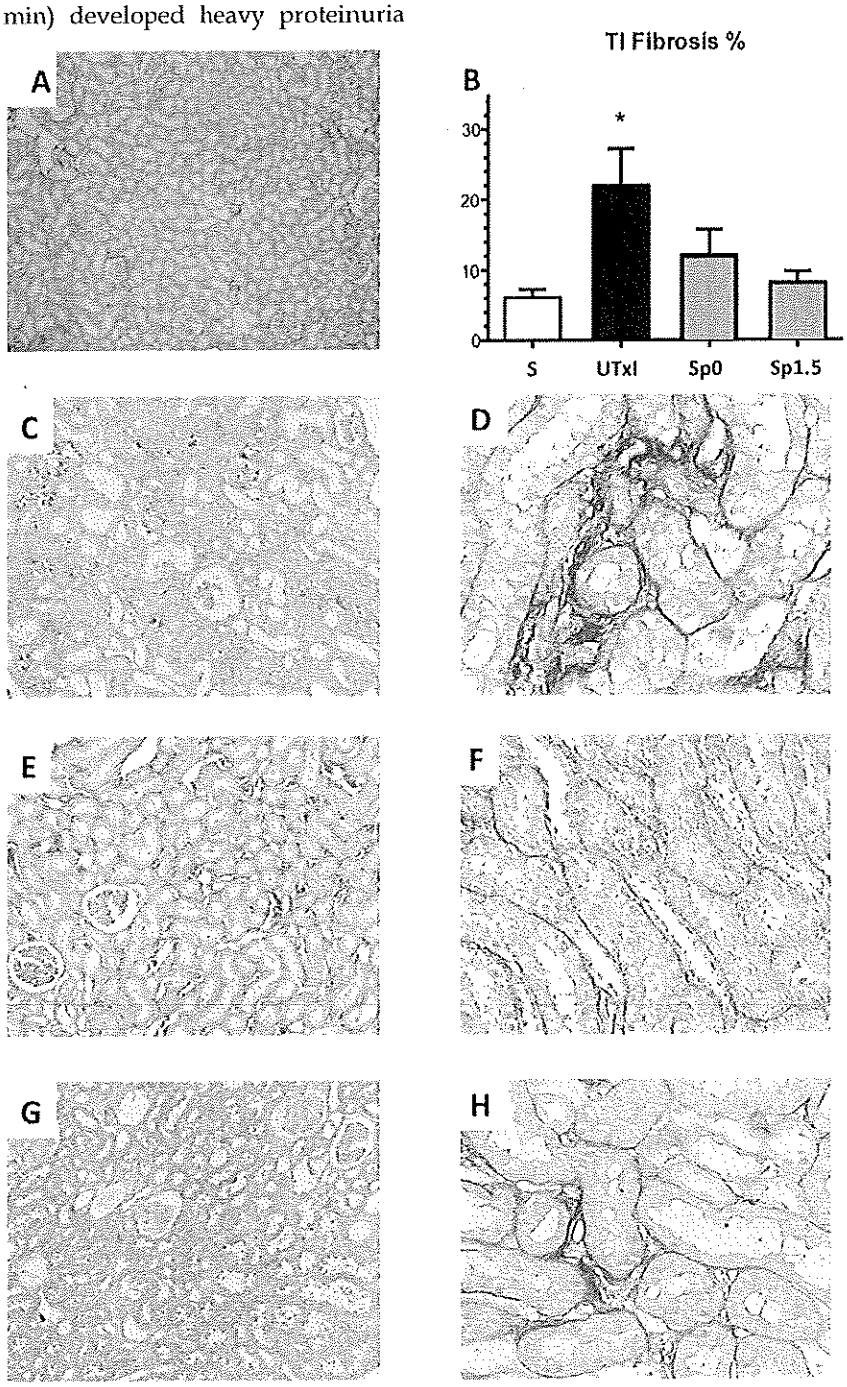

Figure 4. Tubulointerstitial fibrosis development after 9 months of renal ischemia. Representative light micrographs after Sirius red staining showing the presence of fibrosis (in red) from the A) Sham (n=4), C and D) ischemic untreated ischemic group (n=5), E and F) Sp0 (n=4) and G and H) Sp1.5 (n=4). B) The percentage of tubulointerstitial fibrosis in each of the five groups at the end of the 270-day experiment was quantified by morphometric analysis for sham (white bars), untreated ischemic group (black bars), and spironolactone-treated groups (gray bars). Original magnification: X100 (A, C, E, G) and X400 (D, F, H). *$p<0,05$ vs. all the groups.

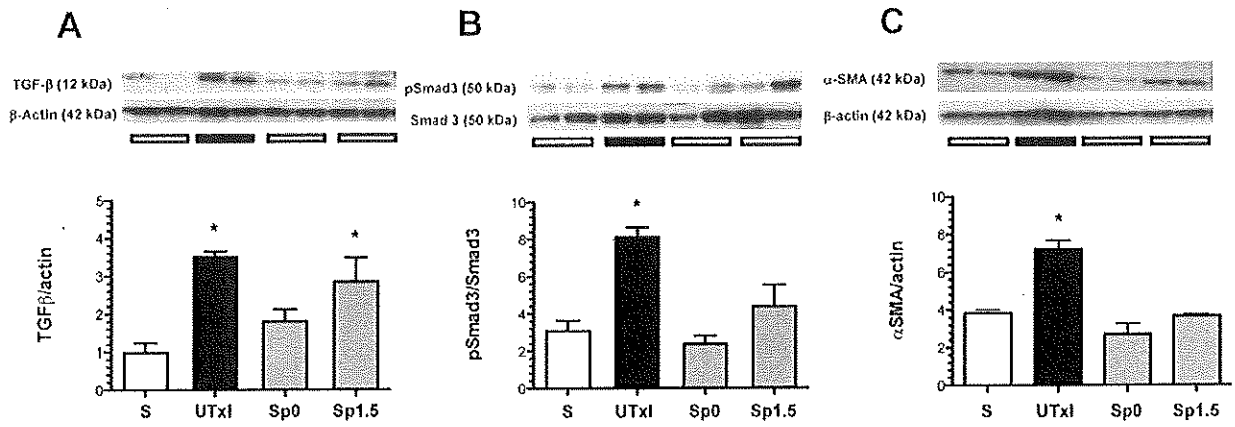

Figure 5. Molecular changes associated with the pro-fibrotic TGF-β pathway activation. A) TGF-β protein levels were quantified by Western blot, B) p-Smad3 and C) α-smooth muscle actin (α-SMA); the densitometric analysis is depicted in the graphs for the sham (white bars, n=4), untreated ischemic group (black bars, n=5), and spironolactone groups (gray bars, n=4). In each panel, the upper insets depict representative blots of the corresponding proteins. *p<0.05 vs. sham operated rats.

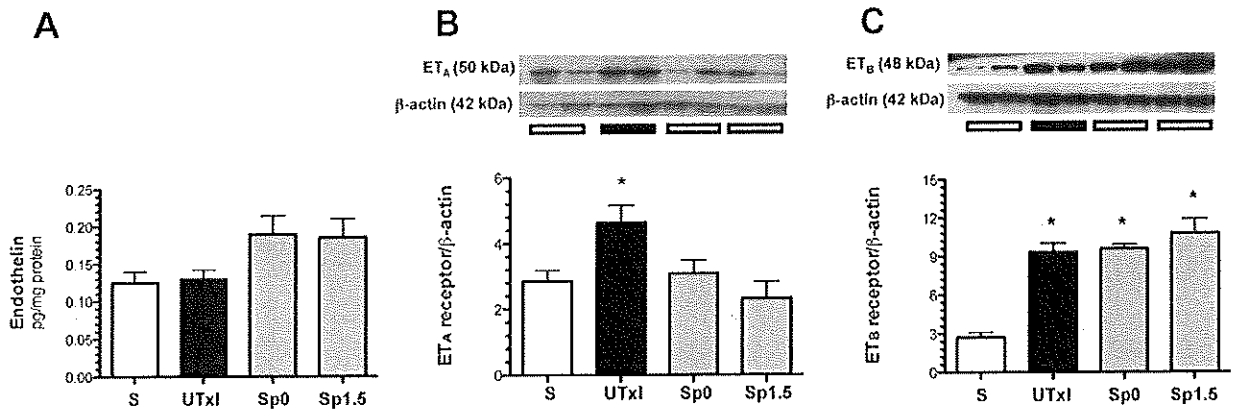

Figure 6. Molecular changes associated with the endothelin pathway. A) Protein levels of endothelin were quantified in renal tissue by an ELISA assay, B) endothelin receptor A (ET_A), and C) endothelin receptor B (ET_B) protein levels. Sham (white bars, n=4), untreated ischemic group (black bars, n=5), and spironolactone-treated groups (gray bars, n=4). * p<0.05 vs. sham operated rats.

In contrast, a brief period of ischemia (10 min) did not lead to proteinuria by the conclusion of the experiment (9 months after ischemia); we cannot exclude the possibility, however, that rats would develop CKD after a longer period of time. This finding is important because many patients in whom AKI may not be accurately diagnosed may be at risk of developing chronic renal injury that may compromise renal function. Indeed, a recent study from Linder A. et al. (30) showed that patients who underwent a mild AKI episode have significantly decreased long-term survival than critically ill patients with no signs of AKI. Although proteinuria developed in rats that underwent 20 min of ischemia, the renal function of the rats was normal nine months after ischemia. These data suggest that the structural injury might be masked by the glomerular compensatory hypertrophy and the nephron functional reserve at this time after mild ischemia. Therefore, although renal dysfunction was not present at this time, these animals exhibited structural and molecular damage, evidenced by glomerular and tubular injury, as well as by effects on pro-fibrotic signaling pathways.

We previously reported that spironolactone administration before or after ischemia may be a useful strategy to prevent the development of CKD induced by a longer period of ischemia. However, this ischemic insult can only be extrapolated to renal transplant or cardiac surgery patients. To investigate a model closer to the common clinical situation, such as patients receiving contrast media or hospitalized patients with lower degree of hypoperfusion, in this study, we investigated spironolactone efficacy in preventing chronic renal injury induced by mild ischemic injury when administered after ischemia. A brief period of ischemia induced structural changes, such as glomerular hypertrophy, glomerulosclerosis, and extensive tubulointerstitial fibrosis. All of these changes were completely prevented by spironolactone administration immediately or up to 1.5-h after

ischemia. Various mechanisms underlying the association between AKI and CKD have been proposed and include chronic hypoxia, abnormalities in the cell cycle of epithelial cells, inflammation, endothelial injury and capillary rarefaction (19;21;29;31). Recently, Kramann et al. (32) showed a reduction in the total number of capillaries and the single capillary area and its perimeter in mice that underwent a severe ischemia, whereas in moderate ischemia, although the number of cortical capillaries did not change, the size of the capillaries was significantly smaller, suggesting that mice with moderate ischemia may also be a target of chronic hypoxia cycles that eventually induce capillary rarefaction after a longer period of time. Indeed, we observed that a mild ischemic insult led to chronic renal injury.

After an ischemic injury, many pathways are activated to promote tubule regeneration; however, some of these pathways remain activated even if the repair is complete, typical of the TGF-β pathway. This mechanism is of particular importance because TGF-β may promote fibroblast trans-differentiation into myofibroblasts and the consequent pro-fibrotic phenotype (33). Indeed, we found that the protein levels of TGF-β, as well as the downstream effector p-Smad3, are increased in rats that underwent a brief period of ischemia. Moreover, an increase in α-SMA, a myofibroblast marker, was also observed. These effects were prevented in the spironolactone-treated rats except for the TGF-β elevation in the Sp1.5 group, however the SMAD-3 phosphorylation was lesser than the untreated ischemic group, suggesting inhibitory SMADs could be inhibiting SMAD-3 phosphorylation.

A recent report suggested that endothelin-1 activation and enhanced ET_A expression may be implicated in the progression from AKI to CKD (18). Therefore, we studied the levels of the endothelin-1, ET_A and ET_B receptors. We could not detect an increase in endothelin-1 levels; however, this discrepancy may be explained by the stage of CKD in which endothelin expression is assessed. In severe CKD, when an extensive area is injured, these cells do not contribute to endothelin production. The differences between rat vs. mice and the effect of bilateral ischemia vs. unilateral ischemia may also account for these results. However, we cannot exclude the idea that endothelin is indeed participating in the progression of CKD, because enhanced expression of the ET_A and ET_B receptors in the rats that underwent mild ischemia was found. Interestingly, the spironolactone treated rats did not present an up-regulation of the ET_A receptor. These data suggest that after ischemia, ET_A remains active and the balance of the receptors may favor the vasoconstrictor effect, which may enhance the chronic hypoxia. In contrast, in spironolactone treated rats, this balance may favor the ET_B receptor, thereby promoting normal perfusion of the kidney and preventing chronic renal injury.

In summary, we provide evidence that in an experimental model of AKI, the duration of ischemia is correlated with the strength and timing of the onset of proteinuria. A mild ischemic lesion was enough to produce progressive proteinuria, renal hypertrophy, glomerular injury, and tubulointerstitial fibrosis. The structural changes were associated with increased TGF-β pathway activation and ET_A receptor up-regulation. These changes were prevented or reduced by spironolactone even if administered after the ischemic insult had occurred. Our data demonstrate the effectiveness of MR antagonism in preventing chronic renal injury induced by a mild ischemic insult in a model of a clinical situation.

Acknowledgments

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Competing Interests

The authors have declared that no competing interest exists.

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AT1 receptor antagonism before ischemia prevents the transition of acute kidney injury to chronic kidney disease

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Despite clinical recovery of patients from an episode of acute kidney injury (AKI), progression to chronic kidney disease (CKD) is possible on long-term follow-up. However, mechanisms of this are poorly understood. Here, we determine whether activation of angiotensin-II type 1 receptors during AKI triggers maladaptive mechanisms that lead to CKD. Nine months after AKI, male Wistar rats develop CKD characterized by renal dysfunction, proteinuria, renal hypertrophy, glomerulosclerosis, tubular atrophy, and tubulointerstitial fibrosis. Renal injury was associated with increased oxidative stress, inflammation, α-smooth muscle actin expression, and activation of transforming growth factor β; the latter mainly found in epithelial cells. Although administration of losartan prior to the initial ischemic insult did not prevent or reduce AKI severity, it effectively prevented eventual CKD. Three days after AKI, renal dysfunction, tubular structural injury, and elevation of urinary biomarkers were present. While the losartan group had similar early renal injury, renal perfusion was completely restored as early as day 3 postischemia. Further, there was increased vascular endothelial growth factor expression and an early activation of hypoxia-inducible factor 1 α, a transcription factor that regulates expression of many genes that help reduce renal injury. Thus, AT1 receptor antagonism prior to ischemia prevented AKI to CKD transition by improving early renal blood flow recovery, lesser inflammation, and increased hypoxia-inducible factor 1 α activity.

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KEYWORDS: glomerular hypertrophy; HIF-1α; inflammation; proximal tubule; renal fibrosis; renal hypoperfusion; VEGF

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Acute kidney injury (AKI) is a common complication in patients who are undergoing major cardiac surgery, experiencing hemorrhage, dehydration, septic shock, diabetes mellitus, or receiving nephrotoxic drugs or contrast media (see review, by Bonventre).[1] The hallmark of ischemic AKI is the reduction in renal blood flow (RBF),[2] provoking endothelial and tubular epithelial injury.[3,4] For many years, it was believed that patients recovering from an AKI episode had no further consequences in the kidney function. However, it is now known that AKI constitutes a risk factor for the development of chronic kidney disease (CKD) and the transition from CKD to end-stage renal disease (ESRD).[5–8] A recent meta-analysis that included 13 major studies with patients who experienced an AKI episode concluded that AKI is an independent risk factor for CKD development.[9] Moreover, from a large number of patients who may suffer an AKI episode throughout their life, it is estimated that 20% would develop CKD in the next 3 years, meaning an estimated 0.3 million patients in higher-income countries and 1.8 million patients in lower-income countries.[10]

During AKI, a number of signaling pathways are activated to repair the affected structures. However, for reasons that are not completely understood, this can lead to cell proliferation, hypertrophy, and disproportionate extracellular matrix production.[4] In the postischemic kidney, renal vasoconstriction is enhanced because of an imbalance of vasoactive substances.[11–13] In addition, the number of vessels in the outer medulla decreases as a result of capillary rarefaction.[14–16] This process can be facilitated by the downregulation of vascular endothelial growth factor.[17] Therefore, the reduction in the number of vessels generates chronic hypoxia that leads to progressive deterioration of the tubular epithelium, leading to cell cycle arrest and epigenetic alterations that eventually cause the progressive development of tubulointerstitial fibrosis.[18,19] Thus, it is essential to delve deeply into the mechanisms by which an AKI episode can trigger an inadequate renal response.

We have recently characterized a model of CKD induced by a single ischemic process in the rat. After recovering from the AKI episode, the animals exhibited progressive

proteinuria, renal dysfunction, and significant histological alterations. Mineralocorticoid receptor blockade before the ischemic insult completely prevented the development of AKI and thus the progression to CKD. Interestingly, spironolactone administration 1–3 h after ischemia also prevented transition from AKI to CKD, implying that activation of the rennin–angiotensin–aldosterone system is involved in the progression to CKD after an AKI episode. We also observed that renal function was completely recovered 10 days after the AKI episode, but signs of inflammation in the kidney persisted that were associated with progression from AKI to CKD along the following months.[20] We previously showed that angiotensin II receptor blockade (ARB) did not prevent AKI induced by unilateral ischemia and because of the cross-talk between vascular and inflammatory effects of angiotensin II, in the present study, we analyzed to what extent ARB with losartan before the ischemic insult was effective in abrogating the severity of the AKI episode and/or the progression to CKD after the AKI episode was resolved.

RESULTS

Figure 1 shows the renal function, structural findings, and biochemical parameters after 24 h of ischemia. Mean arterial pressure was similar among the groups (Figure 1a). Renal injury induced by 45 min of bilateral ischemia was characterized by a significant reduction in the RBF (Figure 1b), reduction in creatinine clearance (Figure 1c), increased serum aldosterone (Figure 1d), elevation of proteinuria (Figure 1e), and severe tubular injury (Figure 1f and 1h). Renal injury was also evidenced by the significant elevation of urinary biomarkers Hsp72 and Kim-1 (Figure 1i and j, respectively). Ischemic renal damage was associated by nitric oxide reduction (Figure 1k) and elevation of oxidative stress (Figure 1l) as we previously reported.[21,22] All of these alterations were not modified by losartan pretreatment, including the elevation of serum aldosterone. Thus, the development of AKI after a single ischemic insult was neither prevented nor reduced by losartan administration.

In another set of experiments, the animals were followed for 9 months after the ischemic insult with and without losartan pretreatment and compared with their respective control groups. As Figure 2a shows in the first 90 days after AKI recovery none of the groups exhibited proteinuria. However, a progressive increase was observed in the untreated ischemic group (UTxI) compared with the sham and losartan control groups. The increased proteinuria was not observed in the animals exposed to ischemia, but previously treated with losartan (Los-Pre), in spite of similar AKI degree (Figure 1a). None of the rats developed hypertension (Figure 2b), as we previously reported.[20] Therefore, all functional and structural alterations were associated with the ischemic process. At the end of the experimental period, UTxI group exhibited a significant reduction in creatinine clearance (Figure 2c), which was accompanied by a slight reduction in RBF. These renal

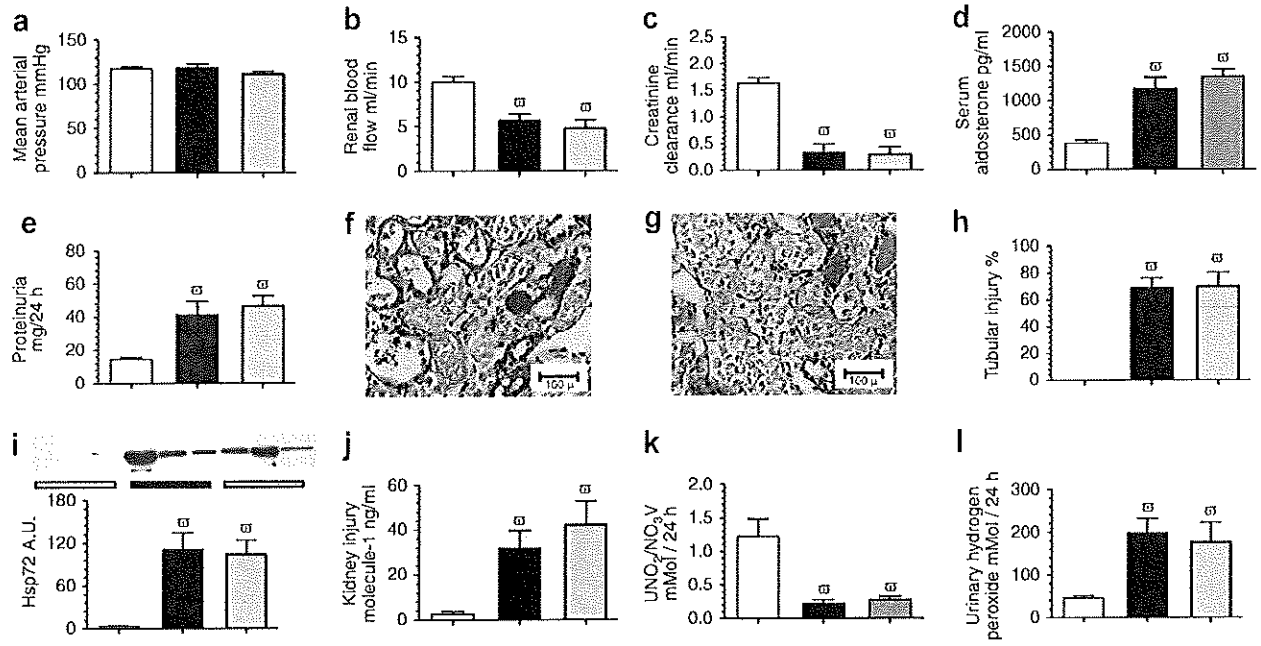

Figure 1 | The prophylactic administration of losartan did not prevent renal injury induced by ischemia/reperfusion. **(a)** Mean arterial pressure, **(b)** renal blood flow, **(c)** creatinine clearance, **(d)** serum aldosterone, **(e)** proteinuria. Sham n = at least 7, UTxI n = at least 6, and Los-Pre n = at least 8 rats; each rat represents one experiment. **(f–g)** Periodic acid–Schiff-stained kidney slides from an UTxI and Los-Pre groups, respectively, **(h)** tubular injury percentage, total tubules taken like 100%, and was determined in at least 5 rats per group. **(i)** Urinary Hsp72 levels by western blot (n = 6 rats per group, one assay), **(j)** urinary Kim-1 excretion (n = 8, one assay), **(k)** urinary NO_2/NO_3 excretion (n = 5, one assay), and **(l)** urinary H_2O_2 excretion (in at least 6 per group, one assay) in sham (white bars), ischemic (black bars), and Los-Pre (gray bars). All parameters were analyzed 24 h after ischemia, and data are shown like mean ± s.e. $^{a}P < 0.05$ versus sham group by analysis of variance and the Bonferroni test.

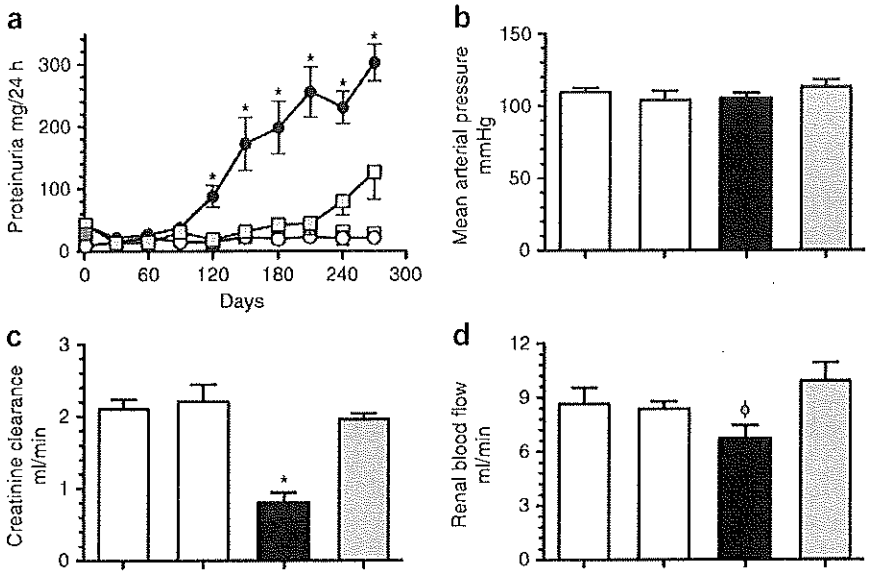

Figure 2 | The prophylactic administration of losartan prevents the progression to chronic kidney disease after an acute kidney injury episode. (a) Urinary protein excretion measured every 30 days during follow-up: open circles represent sham-operated rats (n = at least 6); open squares represent rats that received losartan (50 mg/kg per day) 3 days before sham surgery (n = at least 6); black circles represent rats that underwent renal bilateral ischemia (n = at least 9); gray squares represent rats that received losartan 3 days before renal bilateral ischemia (n = 8). **(b)** Mean arterial pressure (n = at least 6 per group), **(c)** creatinine clearance (n = at least 7 per group), and **(d)** renal blood flow (n = at least 6 per group). All parameters were determined after 9 months in sham (white bars), Los (second white bars), ischemic (black bars), and Los-Pre groups (gray bars). $^{*}P < 0.05$ versus all groups, $^{\Phi}P < 0.05$ versus Los-Pre group, both by analysis of variance and the Bonferroni test.

functional changes were not observed in the ischemic group that received losartan.

Nine months after AKI episode was recovered, histopathological analysis revealed that the UTxI group developed severe structural damage, such as glomerular hypertrophy, tubular atrophy, and cast formation (Figure 3c), compared with control groups (Figure 3a and b). These changes were absent in the Los-Pre group (Figure 3d). Glomerular injury in the UTxI group was confirmed by the glomerulosclerosis percentage (18%), whereas the Los-Pre group was safeguarded from glomerulosclerosis (Figure 3e). A strong correlation between glomerulosclerosis and proteinuria was found (Figure 3f, $P = 0.0001$).

The UTxI group developed significant renal hypertrophy, as their kidney weight was 74% heavier than that of the control group (Supplementary Figure S1A online). The

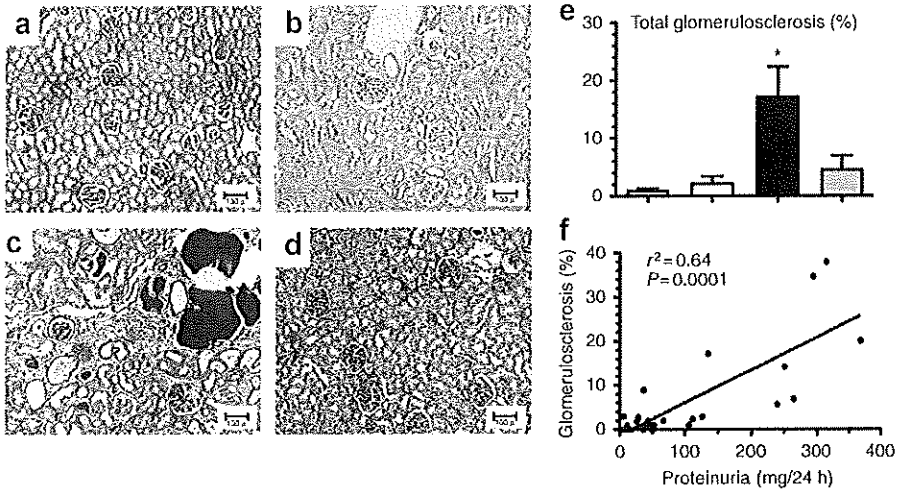

Figure 3 | An acute kidney injury episode leads to extensive structural alterations and prevention by losartan. Representative images of periodic acid–Schiff-stained kidney sections from **(a)** sham-operated, **(b)** Los, **(c)** UTxI, and **(d)** Los-Pre (magnification x100). **(e)** The percentage of glomerulosclerosis was quantified by counting at least 50 glomeruli, which were considered 100% in sham, n = 7 (white bars), Los, n = 8 (second white bars), UTxI, n = 7 (black bars), and Los-Pre, n = 8 (gray bars). **(f)** Pearson correlation between glomerulosclerosis % and urinary protein excretion. $^{*}P < 0.05$ versus all groups by analysis of variance and the Bonferroni test.

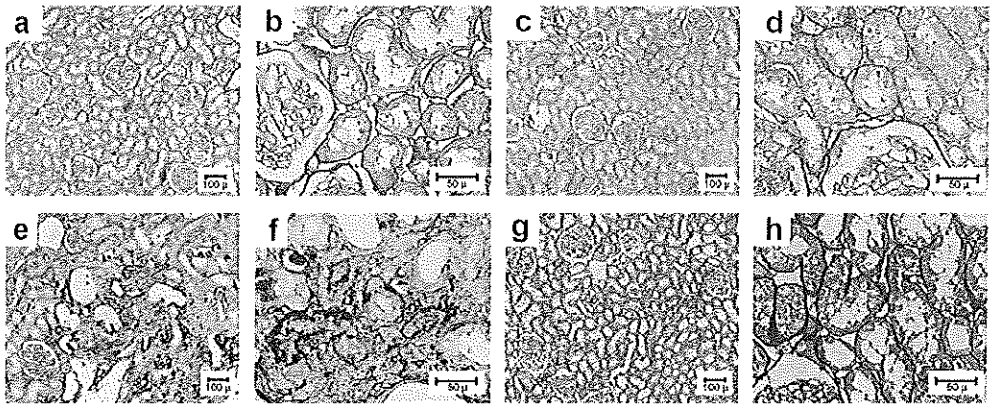

Figure 4 | Chronic kidney disease induced by an acute kidney injury episode was associated with tubulointerstitial injury and prevented by losartan pretreatment. Representative light microphotographs of kidney slides stained with Sirius red from (**a**, **b**) sham-operated, (**c**, **d**) Los, (**e**, **f**) UTxI, and (**g**, **h**) Los-Pre group (magnification ×100 or ×400, respectively).

animals that developed CKD had a higher percentage of glomeruli with diameters greater than 151 μm, compared with the sham or the Los-Pre group (Supplementary Figure S1 online).

An extensive area was affected by tubulointerstitial fibrosis in the UTxI group (Figure 4e and f) compared with the control groups (Figure 4a–d). The Los-Pre group exhibited little staining for Sirus red (Figure 4g and h). These observations were confirmed by the morphometric analysis presented in Figure 5a. Tubulointerstitial fibrosis exhibited a strong correlation with proteinuria (Figure 5b, $P = 0.0001$). Tubular dilation was observed in the UTxI group, exhibiting a 16.7% greater width than the sham-operated group or the Los-Pre group (54.3 ± 1.4 vs. 47.4 ± 1.1, or 49.2 ± 1.6, respectively, $P < 0.05$). In addition, the UTxI group showed higher renal smooth muscle actin (α-SMA) protein levels than the control groups (Figure 5c). In contrast, the ischemic group receiving losartan treatment did not

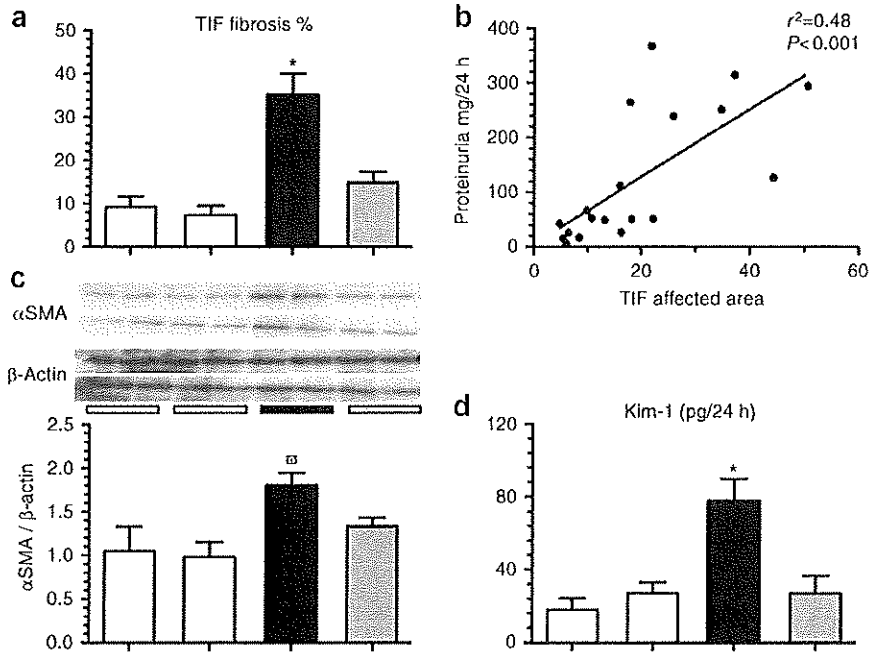

Figure 5 | Involvement of tubule-interstitial fibrosis, proteinuria, and α-smooth muscle actin (SMA) in the acute kidney injury (AKI) transition to chronic kidney disease (CKD). (**a**) Percentage of tubulointerstitial area affected by fibrosis (n = at least 6 per group), evaluated in 10 fields per each rat. (**b**) Pearson correlation between proteinuria and tubulointerstitial fibrosis. (**c**) Insets and densitometric analysis of the western blot of α-SMA and β-actin, respectively ($n = 4$ per group by duplicate). (**d**) Urinary Kim-1 levels (S and Los: $n = 4$, UTxI and Los-Pre: 6 per group, one assay) for sham-operated (white bars), Los (second white bars), UTxI (black bars), and Los-Pre (gray bars). *$P < 0.05$ versus all groups and $^{\varpi}P < 0.05$ versus sham-operated rats by analysis of variance and the Bonferroni test. TIF, tubulointerstital fibrosis.

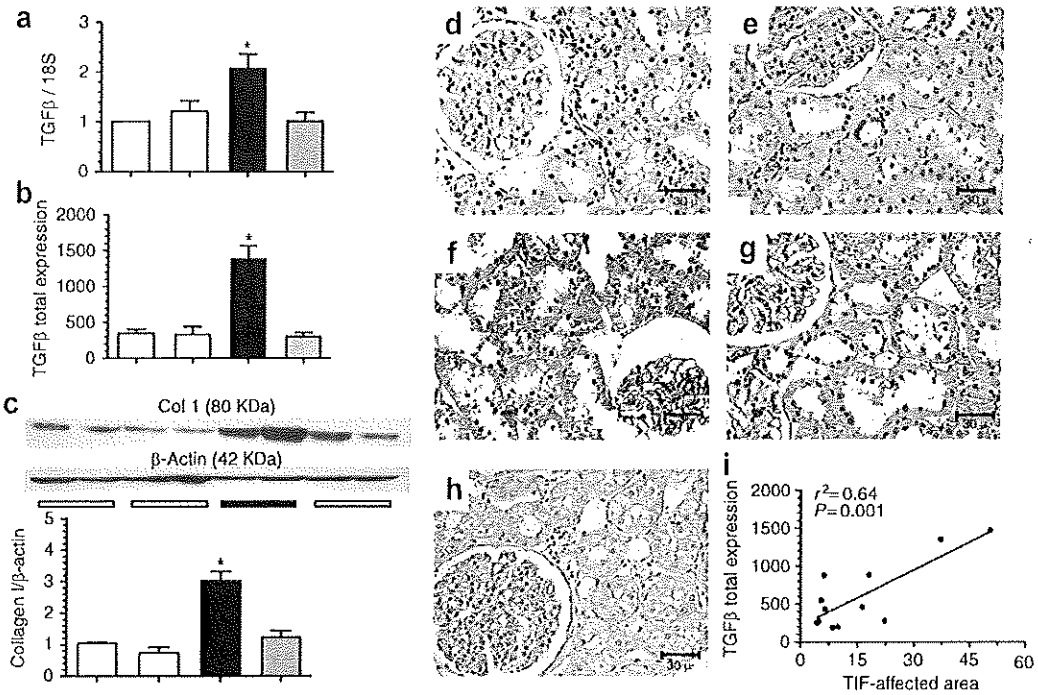

Figure 6 | Pro-fibrotic transforming growth factor-β (TGF-β) cytokine contribution to chronic kidney disease progression. (a) TGF-β mRNA levels were quantified by real-time reverse transcription-polymerase chain reaction. S, $n = 7$; Los, $n = 8$; UTxI, $n = 6$; and Los-Pre group, $n = 7$, by duplicate. **(b)** Renal cortex TGF-β protein levels were assessed by tissue microarray immunohistochemistry and digital image analysis per triplicate in at least 4 rats per group; the protein levels were quantified as total density expression of TGF-β, three different sections per rat. **(c)** Insets and densitometric analysis of the western blot of Col1AI and β-actin, respectively; $n = 4$ per group by duplicate. Representative images of kidney tissue microarray and immunohistochemistry for TGF-β in **(d)** sham-operated, **(e)** Los, **(f)** UTxI, **(g)** Los-Pre (magnification ×400), and **(h)** isotype control. **(i)** Pearson correlation between TGF-β total expression and tubulointerstitial fibrosis. Sham-operated (white bars), Los (second white bars), UTxI (black bars), and Los-Pre (gray bars). *$P < 0.05$ versus all groups by analysis of variance and the Bonferroni test. TIF, tubulointerstital fibrosis.

show this renal α-SMA upregulation. Renal structural damage was also confirmed by urinary kidney injury molecule-1 levels (uKim-1).[23,24] The UTxI group displayed a fourfold increase in uKim-1 (Figure 5d). The renoprotection conferred by prophylactic losartan administration was also demonstrated by the normalization of tubular atrophy and uKim-1.

Figure 6a shows that the renal cortex transforming growth factor-β (TGF-β) mRNA levels were significantly enhanced in the UTxI group, an effect that was reversed by losartan treatment. This finding was corroborated by TGF-β immunohistochemistry from renal microarrays derived from the different studied groups (Figure 6d–g). The UTxI group exhibited greater TGF-β staining, mostly in the tubular epithelium (Figure 6f), compared with the control groups (Figure 6d and e). This staining was not observed in the Los-Pre group (Figure 6g). Accordingly, digital image analysis from these microphotographs revealed a significant upregulation of TGF-β (Figure 6b) in the UTxI group, which was reversed by losartan administration preischemia. As a result of TGF-β activation, collagen I protein levels were significantly enhanced; this effect was not seen in the Los-Pre group (Figure 6c). The influence of TGF-β on renal fibrosis was indicated by the significant correlation between the total expression of TGF-β and tubulointerstitial fibrosis ($P = 0.001$, Figure 6i).

Tubular epithelial proliferation was assessed by immunostaining of proliferating cell nuclear antigen and Ki67. Proliferation was very low in the control groups, as shown by representative microphotographs and by counting positive tubular epithelial cells (Figure 7a–d). In contrast, a significant increase in proliferation was observed in the UTxI group (Figure 7e and g, respectively). In the Los-Pre group, the observed proliferation was similar to the control group (Figure 7f and h, respectively).

All functional and structural alterations observed in the UTxI group were associated with greater oxidative stress, which was assessed by the urinary excretion of H_2O_2 (Supplementary Figure S2A online), in spite of increasing intra-renal G6PD mRNA levels (Supplementary Figure S2B online). Interestingly, oxidative stress enhancement was not observed in the losartan-treated group. Another event involved in the progression to CKD is the activation of inflammation. Consequently, monocyte chemoattractant protein 1 and interleukin-6 mRNA levels were upregulated in the UTxI group (Supplementary Figure S2C–D online). This

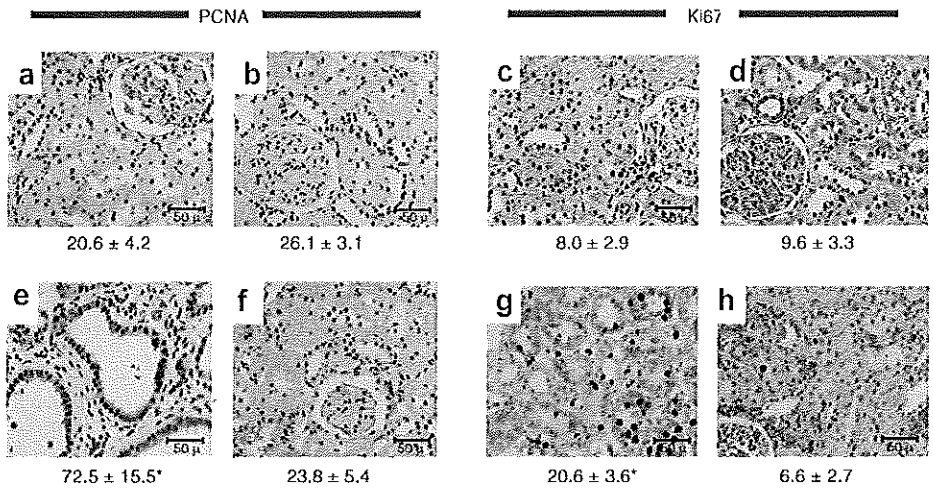

Figure 7 | Tubular atrophy observed in rats with chronic kidney disease was associated with epithelial cell proliferation and preserved by losartan. Tubular proliferation was assessed by proliferating cell nuclear antigen (PCNA) and Ki67 immunohistochemistry as shown in the representative microphotographs from kidney slides (magnification ×400). **(a, c)** Sham-operated rats. **(b, d)** Los. **(e, g)** UTxI. **(f, h)** Los pre. The mean ± SD of PCNA and Ki67-positive epithelial cells is shown under the corresponding image. *$P < 0.05$ versus all groups by analysis of variance and the Bonferroni test.

pattern was not observed in the Los-Pre group, showing a state of minor inflammation.

To determine the mechanisms by which losartan prevented the AKI to CKD transition, although AKI development was not prevented, we studied a set of rats in an early stage postischemia.

In Figure 8 appears the physiological and biochemical results at 3 days postischemia. We found that, in the UTxI group, the renal hypoperfusion and dysfunction persisted (Figure 8b and c) without proteinuria (Figure 8d). At the structural level, tubular injury was evident (Figure 8e) and correlated with the elevation of urinary Hsp72 and Kim-1 (Figure 8g and h, respectively). All these alterations were similarly observed in the Los-Pre group, except in the early recovery of RBF (Figure 8b).

Figure 9 shows the physiological, biochemical, and molecular findings at 5 days postischemia. We found that, in the UTxI group, the renal dysfunction lasted (Figure 9b and 9c) without proteinuria (Figure 9d), but with urinary Kim-1 that persisted elevated (Figure 9e). All these abnormalities were not seen in Los-Pre group. Although urinary H_2O_2 elevation in the UTxI group did not reach statistical difference

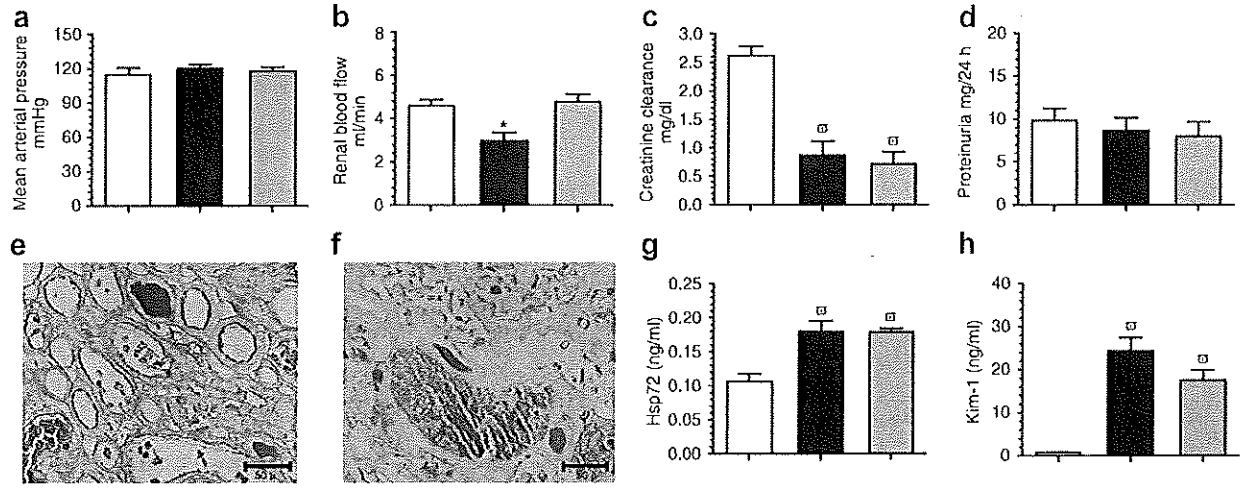

Figure 8 | Renal dysfunction and structural injury persist after 3 days of ischemia, and losartan only prevented renal hypoperfusion. (a) Mean arterial pressure, **(b)** renal blood flow, **(c)** creatinine clearance, **(d)** proteinuria, **(e)** periodic acid–Schiff-stained kidney slides from an UTxI and **(f)** Los-Pre groups, respectively; **(g)** urinary Hsp72 levels and **(h)** urinary Kim-1 excretion. Sham operated is represented by white bars, n = at least 5; UTxI is represented by black bars, n = at least 6 rats; and Los-Pre is represented by gray bars, n = at least 5. *$P < 0.05$ versus all groups, $^{\alpha}P < 0.05$ versus sham, by analysis of variance and the Bonferroni test.

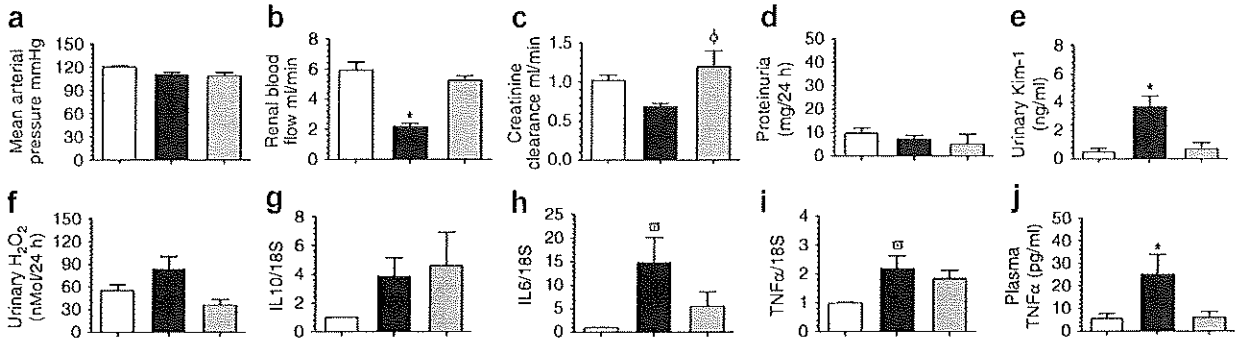

Figure 9 | The renal dysfunction and inflammation persist after 5 days of ischemia but not in losartan pretreated rats. **(a)** Mean arterial pressure, **(b)** renal blood flow, **(c)** creatinine clearance, **(d)** proteinuria, **(e)** urinary Kim-1 excretion, **(f)** urinary H_2O_2 excretion, **(g)** interleukin (IL)-10 mRNA levels, **(h)** IL-6 mRNA levels, **(i)** tumor necrosis factor (TNF)-α mRNA levels, and **(j)** plasma TNF-α levels. Physiological parameters and plasma TNF-α levels were determined once, whereas mRNA levels were determined at least by duplicate. Sham operated is represented by white bars, $n = 5$; UTxI is represented by black bars, $n = 5$; and Los-Pre is represented by gray bars, $n = 4$. All parameters were analyzed 5 days after ischemia. $^{*}P < 0.05$ versus all groups, $^{\text{m}}P < 0.05$ versus sham, and $^{\Phi}P < 0.05$ versus Los-Pre by analysis of variance and the Bonferroni test.

(Figure 9f), interlukin-6 and tumor necrosis factor-α were significantly elevated (Figure 9h–j). Interestingly, the faster recovery of the renal dysfunction in the Los-Pre group was associated with normalization of urinary H_2O_2 excretion and inflammatory cytokines expression.

HIF-1α is a transcription factor that promotes the transcription of genes necessary for the survival of the cell when there is a drop in the oxygen supply. Renal HIF-1α mRNA levels were similar among the groups and were not modified after 1, 5 (Supplementary Figure S3 online), or 15 days post-ischemia (Figure 10g). In contrast, the total and nuclear HIF-1α protein levels, measured by tissue microarray in the kidney cortex and medulla, increased significantly after 15 days in the Los-Pre group (Figure 10e–f, respectively, and 10i–k, respectively), an effect that was not observed in the UTxI group (Figure 10c, d and k). Most of the expression was localized into the tubular epithelium. To evaluate the HIF-1α nuclear transcriptional activity, the protein levels of vascular

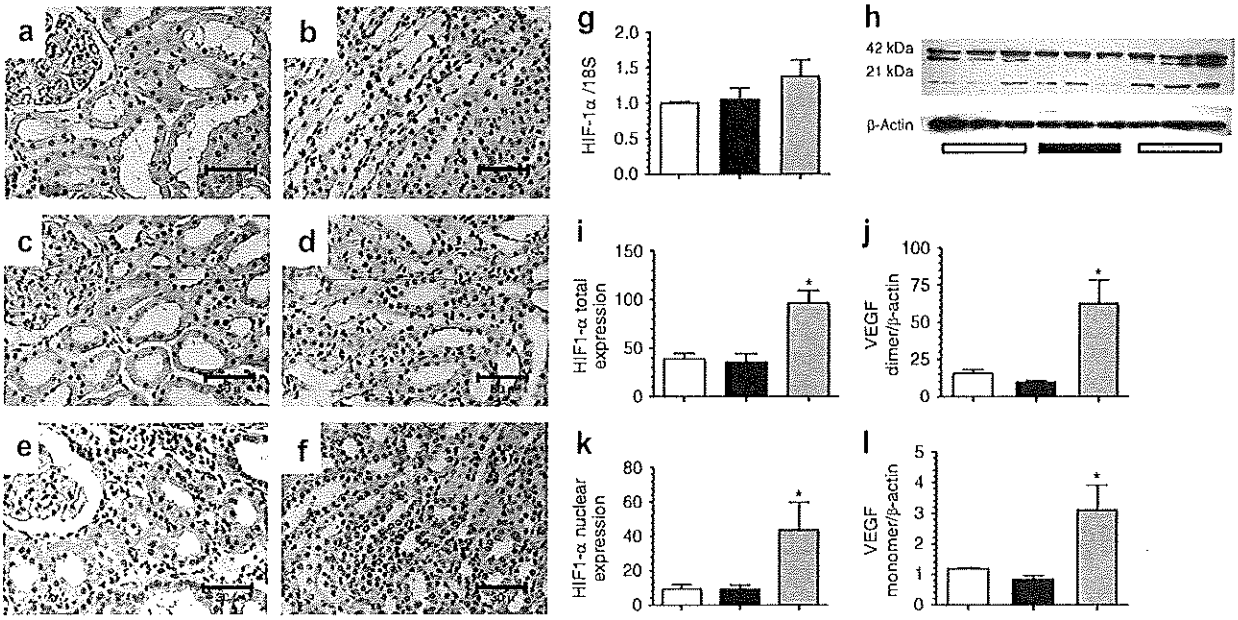

Figure 10 | HIF-1α and VEGF in the renal cortex after an ischemic event. HIF-1α/protein was assessed after 15 days of ischemia by tissue microarray immunohistochemistry and digital image analysis per triplicate in at least 4 rats per group in renal cortex **(a, c,** and **e)** and in renal medulla **(b, d,** and **f)**. **(a, b)** Representative microphotographs of the sham group, **(c** and **d)** the UTx group, and the **(e** and **f)** Los-Pre group. **(g)** HIF-1α mRNA levels after 15 days of ischemia, **(i)** total density expression of HIF-1α, three different sections per rat tissue microarray, and **(k)** nuclear HIF-1α/protein. **(h)** Insets of the western blot of VEGF and β-actin, respectively; $n = 4$ per group by duplicate. **(j** and **l)** VEGF/β-actin densitometric analysis for dimer and monomer VEGF conformation, respectively. Sham operated is represented by white bars; UTxI is represented by black bars; and Los-Pre is represented by gray bars. $^{*}P < 0.05$ versus all groups by analysis of variance and the Bonferroni test. VEGF, vascular endothelial growth factor. HIF, hypoxia-inducible factor.

endothelial growth factor (VEGF) were assessed after 15 days postischemia. Western blot analysis revealed two bands corresponding to the monomer and dimer VEGF conformation (Figure 10h). The densitometric analysis of both dimer and monomer (Figure 10j and l, respectively) shows a slight reduction in the UTx group, but the differences were not significant by analysis of variance. According to the greater nuclear HIF-1α observed in the Los-Pre group, monomer and dimer VEGF was significantly enhanced (Figure 10h, j and l).

DISCUSSION

In this study, we have investigated the mechanisms that lead to CKD induced by a single AKI episode, and we have also provided evidence of the importance of an early intervention, the prophylactic administration of losartan, for stopping or slowing down CKD progression. Our data show that, although losartan pretreatment did not protect the rats against AKI, it was effective to prevent the transition to CKD. The UTxI group developed CKD characterized by renal hypertrophy, renal dysfunction, glomerular hypertrophy, glomerulosclerosis, tubular atrophy, and tubulointerstitial fibrosis. These functional and structural alterations were associated with increased α-SMA protein levels, oxidative stress, inflammation, and activation of TGF-β.

Previously, we showed that angiotensin II blockade with a low dose of losartan (8 mg/K) did not prevent renal injury induced by unilateral ischemia, and only a higher dose (80 mg/K) had a minor effect.[25] Here, we showed that prophylactic losartan administration did not prevent AKI induced by severe bilateral renal ischemia. However, after 9 months, the Los-Pre group preserved an adequate renal function, as well as glomerular and tubular architecture.

Under hypoxic conditions, the transcription factor HIF-1α has a crucial role in regulating the expression of more than 100 target genes involved in cell proliferation, angiogenesis, glucose metabolism, and apoptosis, among others (see review by Shoji *et al.*[26]). HIF-1α expression is regulated by proteosomal degradation mediated by prolyl hydroxylase domain (PHD) containing protein enzymes. Indeed, PHD inhibitors not only induced HIF-1α activation but also reduced renal injury induced by ischemia/reperfusion,[27] subtotal nephrectomy,[28] allogenic kidney transplant,[29] or nephritis.[30] These data suggest that HIF-1α exerts a protective role under AKI. In this study, we found that HIF-1α mRNA levels were not different among the groups after 1, 5, or 15 days postischemia, but the total and nuclear HIF-1α expression assessed by tissue microarray was significantly enhanced in the Los-Pre group after 15 days of ischemia. This activation could result from AT1 receptor inhibition by losartan, because it has been proposed that PHD inhibition induces AT1 receptor downregulation and a lesser perivascular fibrosis in coronary arteries.[31] Our data also suggest that after an ischemic insult there is an ineffective HIF-1α activation to face the renal hypoperfusion, vascular rarefaction, and inflammation. In contrast, losartan treatment was able to induce HIF-1α nuclear translocation and activation, which was detected by inducing VEGF transcription. Our data suggest that VEGF could diminish capillary rarefaction, improve renal perfusion, and reduce chronic hypoxia. Although we cannot explain how ARB induced HIF-1α after 15 days of ischemia, it could result by inducing one of the following: lesser HIF-1α degradation, greater HIF-1α/HIF-1β dimerization, or enhanced nuclear translocation. Our results show that the HIF-1α activation after ischemia was associated with the prevention of AKI to CKD transition, however, the mechanism by which ARB activates HIF-1α remained to be explored.

After 9 months, the persistent proteinuria in UTxI group correlated with glomerulosclerosis and tubulointerstitial fibrosis, suggesting that abnormal proteinuria is due to gradual damage to both the glomerular filtration barrier, by diffuse podocyte effacement and reduction in nephrin expression (data not shown), and tubular epithelium atrophy and dilation after an AKI episode. This picture is very similar to that observed in the recently characterized Mesoamerican nephropathy, in which affected patients were normotensive but exhibited glomerular enlargement, glomerulosclerosis, chronic tubulointerstitial injury, tubular atrophy, and interstitial fibrosis.[32] Thus, our findings support the hypothesis that this nephropathy is related with one or several episodes of AKI.[32]

Renal ischemic injury has been associated with atubular nephrons.[33] Therefore, it is likely that, in the first weeks postischemia, a significant number of nephrons are lost. The nephron reduction means that the remaining functional nephrons must compensate for the lost function through hyperfiltration and hypertrophy. Indeed, renal hypertrophy was evident in the UTxI group and avoided by prophylactic losartan administration. A recent study showed that renal hypertrophy induced by ischemia depends on the extent of tubular epithelium death mediated by tumor necrosis factor-α signaling pathway activation.[34] These abnormalities certainly accelerate the deterioration of the functional nephrons. Thus, it feasible that the Los-Pre group had lesser atubular nephrons, which is reflected by better renal function and glomerular structure preservation. Indeed, Pagtalunan *et al.*[35] showed that losartan postischemia was able to reduce the number of atubular nephrons.

Proximal tubular epithelial cells suffer death by necrosis or apoptosis, and others lose their polarity and slough off after an AKI episode. Consequently, epithelial dedifferentiation and proliferation are triggered with the purpose of restoring the tubular epithelium. Tubular repair, however, is altered by processes such as cell arrest[18] and epigenetic changes[19] that instead of improving renal architecture switch on fibrogenesis. Our study showed that the UTxI group exhibited an atrophic tubular epithelium that was associated with increased proliferation, assessed by Ki67 and proliferating cell nuclear antigen. Similar results have been observed in renal biopsies from patients suffering AKI.[36] These results indicate that tubular proliferation is

perpetuated as a maladaptive phenomenon, which was not seen in the Los-Pre group.

Progressive tubulointerstitial fibrosis is a key player in chronic renal injury and involves chronic peritubular inflammation, tubular cell dedifferentiation, and myofibroblast activation.[37,38] Furthermore, sustained leukocyte accumulation and activation inside the kidney could extend periods of ischemia due to vascular congestion and may induce direct tubular and endothelial cell damage by the release of inflammatory mediators.[39] In our study, we observed that an ischemic insult promoted an extensive tubulointerstitial area affected by fibrosis. This alteration was, in part, mediated by TGF-β upregulation. Yang *et al.*[18] proposed that, after a severe ischemic insult, the tubular epithelium produces large amounts of TGF-β. Our results with tissue microarray confirmed these previous findings; TGF-β was upregulated, and most of the TGF-β immunostaining was found in the tubular epithelium. The fibrotic response was not observed in Los-Pre group. Bechtel *et al.*[19] have shown that injured epithelial cells trigger phenotypical modulation of fibroblasts to activated myofibroblasts. Accordingly, we observed that renal α-SMA levels, a marker of fibroblast transdifferentiation, were increased in the UTxI group, and this effect was prevented in the Los-Pre group.

Our data suggest that AT_1 receptors must be blocked at the moment of the ischemic insult to avoid angiotensin-II involvement in the ineffective endothelial and tubular reparation, which occurred in long term. In this regard, and because AKI occurs in 30% of patients undergoing cardiac surgery, several clinical studies have examined the effect of angiotensin-converting enzyme inhibitors (ACEis) or ARB on AKI incidence. However, inconclusive results have been published on AKI incidence in cardiovascular surgery: increased,[40] or no change,[41,42] or even reduced.[43,44] Recently, Coca *et al.*[45] studied the effect of held or continued ACEi/ARB treatment in patients undergoing cardiac surgery compared with patients not treated with ACEi/ARB and demonstrated similar levels of urinary biomarkers such as NGAL, interlukin-18, Kim-1, and liver-type fatty acid binding protein among the patients and a reduction in the glomerular filtration rate in the group that continued with ACEi/ARB therapy. Furthermore, glomerular filtration rate reduction has not been attributed as an adverse effect because these drugs improve peritubular capillary perfusion, which in turn could reduce tubular ischemia and necrosis.[46–48] Unfortunately, long-term evaluation of patients suffering from AKI and receiving ACEi or ARB therapy has not been reported.

Previously, we showed that prophylactic administration of spironolactone completely prevented AKI and the transition to CKD.[20] In this study, losartan pretreatment before ischemia did not prevent either aldosterone elevation or AKI severity within 24-h postinsult, demonstrating that ARB protection was independent of MR receptor antagonism and suggesting that aldosterone and angiotensin II differentially regulate both renal hemodynamics and the involvement of signal pathways for endothelial and tubular reparation.

Our findings not only reinforce the great importance of an AKI episode as a risk factor for the development of CKD but also show the deleterious effect of the activation of AT1 receptors during an ischemic insult and its impact on the lasting renal function and structure. This study also shows the potentiality of ARB in preventing the AKI transition to CKD by a mechanism related with the early recovery of RBF, prevention of an inflammatory process, enhanced HIF-1α nuclear translocation, and early induction of VEGF after the ischemic insult. Timely identification of AKI, together with an effective prophylactic intervention, would have a marked impact in slowing down CKD progression.

MATERIALS AND METHODS

All experiments involving animals were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (https://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-use-of-laboratory-animals.pdf) and were approved by the Animal Care and Use Committee at our Institutions.

Thirty-four male Wistar rats weighing 250–300 g were divided into four groups—(i) sham-operated rats, $n = 8$ (S); (ii) sham-operated rats plus losartan (50 mg/kg/day by gastric gavage) 3 days before surgery, $n = 8$ (Los); (iii) rats who underwent bilateral renal ischemia for 45 min, $n = 9$(UTxI), and (iv) group that received losartan 3 days before bilateral renal ischemia, $n = 9$ (Los-Pre)—and were observed for 9 months. In another set of experiments, 21 rats from S, 26 from UtxI, and 25 from Los-Pre groups were studied and divided into four different periods: 1, 3, 5, or 15 days after ischemia. All animals were kept in a 12:12 h day–night cycle and had free access to water and food.

Ischemia/reperfusion model

Rats were anesthetized with an intra-peritoneal injection of sodium pentobarbital (30 mg/kg) and placed on a heating pad to maintain core body temperature at 37 °C. Renal pedicles were isolated, and bilateral renal ischemia was induced using a non-traumatic clamp on each renal artery for 45 min. Ischemia was verified visually by a change in the kidney color. Reperfusion was achieved by release of the clips and confirmed by return of oxygenated blood to the kidney. The incision in the muscle and the skin was closed with 3-0 vicryl and silk sutures, respectively. For sham surgery, laparotomy and renal pedicle dissection, without clamping, were performed in anesthetized rats.

Statistical analysis

The results are presented as the mean ± s.e. The significance of the differences between groups was assessed by analysis of variance using the Bonferroni correction for multiple comparisons. All comparisons passed the normality test. The differences in the ranks of glomerular diameters among the groups were evaluated by contingency analysis, and the differences were assessed using the χ^2 test with the Yates correction. The correlation among the data was evaluated by Pearson's test. Statistical significance was defined when the P value was < 0.05.

More detailed Methods appear as Supplementary Material.

DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTION

RRR and NAB: conceived and design the study. RRR, KB, JBC, RPV, AG, and NAB: performed the experiments, molecular, biochemical, and histopathological analysis. NU and DA: histology and immunohistochemistry. SH and JFRS: immunohistochemistry by microarrays. RRR and NAB: analyzed the data. NAB: contributed reagents or analysis tools. RRR, GG, and NAB: wrote the article.

SUPPLEMENTARY MATERIAL

Figure S1. Renal and glomerular hypertrophy induced by an AKI episode was prevented by losartan administration.

Figure S2. Oxidative stress and inflammation responses in CKD group were inhibited with losartan administration before ischemic insult.

Figure S3. HIF-1α mRNA and protein levels in the renal cortex after 1 and 5 days of ischemia.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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VNIVERSIDAD NACIONAL
AVPNOMA DE MEXICO

ACTA DE EXAMEN

Ciudad Universitaria, Cd. de México., a los 5 días del mes de mayo de 2016, se celebró el examen para obtener el grado de **DOCTORA EN CIENCIAS**, que sustentó **ROXANA MINERVA RODRÍGUEZ ROMO**, de nacionalidad mexicana, registrada con el número de cuenta **98277489**, quien cursó los estudios en el periodo comprendido de **2012 a 2015-2** y cumplió con los requisitos académicos señalados en el plan de estudio correspondiente, habiendo presentado la tesis: **"IMPLICACIÓN DE ANGIOTENSINA II EN LA PROGRESIÓN A ENFERMEDAD RENAL CRÓNICA (ERC) COMO CONSECUENCIA DE LA LESIÓN RENAL AGUDA"**
Dirigida por la Dra. Norma Araceli Bobadilla Sandoval

El Comité Académico del Programa designó el jurado, formado por los profesores que a continuación se mencionan y que fungieron como:

Presidente: Dra. María Eugenia Gonsebatt Bonaparte

Vocal: Dra. Marcia Hiriart Urdanivia

Vocal: _____

Vocal: _____

Secretario: Dra. Norma Araceli Bobadilla Sandoval

Al término del examen el jurado resolvió: Aprobarla con mención honorífica.

Se dio por concluido el acto académico con las firmas de los sinodales que en él intervinieron.

Presidente **Secretario**

Marcia Hiriart U. **Vocal** _____ **Vocal** _____ **Vocal**

"POR MI RAZA HABLARÁ EL ESPÍRITU"

La suscrita, Coordinadora del Programa de Doctorado en Ciencias Biomédicas, certifica que las firmas son auténticas y corresponden al jurado designado.

DRA. AUREA OROZCO RIVAS
COORDINADORA

Por la Dirección General de Administración Escolar

Jefe del Departamento de Exámenes y Títulos Subdirector de Control Documental

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VNIVERSIDAD NACIONAL
AVPNOMA DE MEXICO

CONSTANCIA DE
EXAMEN

Dra. Norma A. Bobadilla Sandoval, secretario del jurado
que examinó a Roxana Minerva Rodríguez Romo
para optar por el grado de Doctora
en Ciencias
hace constar que obtuvo la calificación de Aprobada
con Mención Honorífica.

Ciudad Universitaria, Cd. de Mx., a 5 de mayo de 2016.

EL SECRETARIO DEL JURADO

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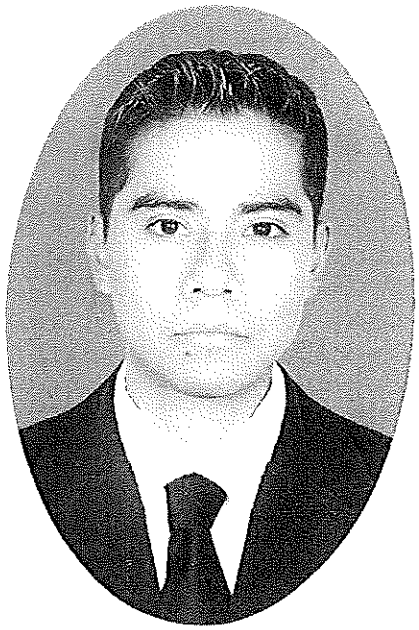
**Al cabo de 50 días hábiles posteriores al Examen de Grado
Deberá consultar la página con tu número de cuenta y nip, para checar el avance de emisión del título o grado. "Tramitel" ubicado en el Edificio de la D. G. A. E., Circuito de la Investigación Científica entre la parada C. U. del metro Universidad y el Centro de Desarrollo Infantil (CENDI), en Ciudad Universitaria.**

VNIVERSIDAD NACIONAL AVTÓNOMA DE MEXICO

ACTA DE EXAMEN



México, Distrito Federal, a los 9 días del mes de mayo de 2013, se celebró el examen para obtener el grado de **DOCTOR EN CIENCIAS**, que sustentó **JONATAN BARRERA CHIMAL**, de nacionalidad mexicana, registrado con el número de cuenta **303114288,** quien cursó los estudios en el periodo comprendido de **2010 a 2013**, y cumplió con los requisitos académicos señalados en el plan de estudio correspondiente, habiendo presentado la tesis **"ESTUDIO DE LOS MECANISMOS INVOLUCRADOS EN LA LESIÓN RENAL AGUDA Y SU PROGRESIÓN A ENFERMEDAD RENAL CRÓNICA: DIAGNÓSTICO Y TERAPÉUTICA"**
Dirigida por la: Dra. Norma Araceli Bobadilla Sandoval

El Comité Académico del Programa designó el jurado, formado por los profesores que a continuación se mencionan y que fungieron como:

Presidente: Dr. Federico Martínez Montes

Vocal: Dr. Juan Carlos Gómora Martínez

Vocal: _____

Vocal: _____

Secretario: Dra. Norma Araceli Bobadilla Sandoval

Al término del examen el jurado resolvió: Aprobarlo con Mención Honorífica

Se dio por concluido el acto académico con las firmas de los sinodales que en él intervinieron.

Presidente	**Secretario**	
Vocal	**Vocal**	**Vocal**

"POR MI RAZA HABLARÁ EL ESPÍRITU"

El suscrito, Coordinador del Programa de Doctorado en Ciencias Biomédicas, certifica que las firmas son auténticas y corresponden al jurado designado.

DR. DANIEL PIÑERO DALMAU
COORDINADOR

Por la Dirección General de Administración Escolar

Jefe del Departamento de Exámenes y Títulos Subdirector de Control Documental

5002078213034

UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

DOCTORADO EN CIENCIAS BIOMÉDICAS

INSTITUTO DE INVESTIGACIONES BIOMÉDICAS

ESTUDIO DE LOS MECANISMOS INVOLUCRADOS EN LA LESIÓN RENAL AGUDA Y SU PROGRESIÓN A ENFERMEDAD RENAL CRÓNICA: DIAGNÓSTICO Y TERAPÉUTICA

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTOR EN CIENCIAS

PRESENTA:

JONATAN BARRERA CHIMAL

DIRECTOR DE TESIS:

DRA. NORMA ARACELI BOBADILLA SANDOVAL

INSTITUTO DE INVESTIGACIONES BIOMÉDICAS

COMITÉ TUTOR:

DRA. MARÍA EUGENIA GONSEBATT BONAPARTE

INSTITUTO DE INVESTIGACIONES BIOMÉDICAS

DR. JULIO EDUARDO ROQUE MORÁN ANDRADE

INSTITUTO DE FISIOLOGÍA CÉLULAR

MÉXICO, D. F. MAYO DE 2013

UNIVERSIDAD NACIONAL AUTÓNOMA DE MEXICO
DOCTORADO EN CIENCIAS BIOMÉDICAS

IMPLICACIÓN DE ANGIOTENSINA II EN LA PROGRESIÓN A ENFERMEDAD RENAL CRÓNICA (ERC) COMO CONSECUENCIA DE LA LESIÓN RENAL AGUDA.

TESIS
QUE PARA OPTAR POR EL GRADO DE:
DOCTOR EN CIENCIAS

PRESENTA: RODRÍGUEZ ROMO ROXANA MINERVA

TUTOR PRINCIPAL

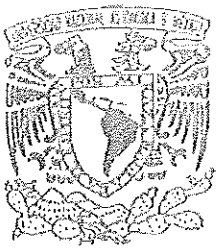
DRA. NORMA A. BOBADILLA SANDOVAL
INSTITUTO DE INVESTIGACIONES BIOMÉDICAS

Comité Tutoral

Dra. Elena Zambrano González
Facultad de Medicina

Dra. Carmen Clapp Jiménez Labora
Instituto de Neurobiología

Ciudad de México, Mayo 2015



UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

FACULTAD DE CIENCIAS

ALTERACIONES HISTOLÓGICAS EN LA PROGRESIÓN A ENFERMEDAD RENAL CRÓNICA SECUNDARIA A UN EVENTO DE LESIÓN RENAL AGUDA

T E S I S

QUE PARA OBTENER EL TÍTULO DE:

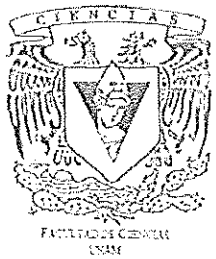
BIÓLOGO

P R E S E N T A:

ARTURO GÓMEZ ROMERO

TUTORA:

DRA. NORMA ARACELI BOBADILLA SANDOVAL



2015

Facultad De Ciencias
Secretaría General
División de Estudios Profesionales

Constancia de Examen Profesional

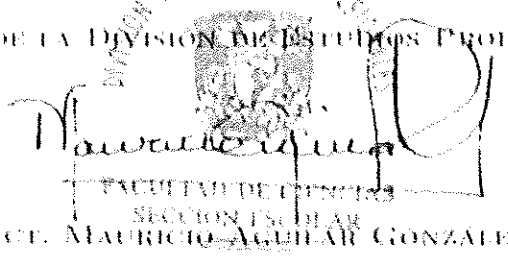
A quien corresponda

Por este medio se hace constar que Arturo Gómez Romero, alumno de la carrera de Biología que se imparte en esta Facultad, sustentó y aprobó su examen profesional el día 7 de abril de 2016

Cabe agregar que el mencionado deberá continuar los trámites necesarios ante la Dirección General de Administración Escolar de la UNAM y la Secretaría de Educación Pública, para obtener su título y cédula profesional

Atentamente
"Por Mi Raza Hablará El Espíritu"
Ciudad Universitaria D. F., a 7 de abril de 2016

El Jefe de la División de Estudios Profesionales



Act. Mauricio Aguilar González

INSTITUTO NACIONAL DE
CIENCIAS MÉDICAS
Y NUTRICIÓN
SALVADOR ZUBIRÁN

México, Cd.Mx., a 8 de Mayo del 2018

Dr. Jorge Barrios Payan
Secretario de la CINVA
P R E S E N T E .

Estimado Dr. Barrios:

Por este conducto me permito solicitar el cierre del protocolo: "**Implicación de la Aldosterona en el desarrollo de enfermedad renal crónica como consecuencia de una lesión renal aguda**" con registro **CINVA: NMN-45-11-13-1,** debido a que el protocolo ha concluido.

Sin otro particular por el momento, quedo de usted.

Atentamente,

Dra. Norma A. Bobadilla Sandoval
Investigador en Ciencia Médicas F
Depto. Nefrología y Metabolismo Mineral

Avenida Vasco de
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Acuse

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN

México Cd., Mx a 26 de abril de 2018.

No. Oficio CINVA 048-18

Dra. Norma A. Bobadilla Sandoval
Depto. Nefrología y Metabolismo Mineral
P r e s e n t e.

Estimada Dra. Bobadilla.:

Por este conducto le informo que su proyecto con título **"IMPLICACIÓN DE LA ALDOSTERONA EN EL DESARROLLO DE ENFERMEDAD RENAL CRÓNICA COMO CONSECUENCIA DE UNA LESIÓN RENAL AGUDA",** con registro **CINVA NMM-45-11-13-1** finalizó en julio 2017. Debido a que el periodo de realización y la prórroga han concluido, le solicito de la manera más atenta favor de llenar el formato de cierre del proyecto que se anexa a la presente (en hoja membretada e impresa) y adjunte los siguientes documentos indispensables para la conclusión del proyecto:

1. Informe final
2. Productos de Investigación derivados del proyecto (artículos, tesis, libros, capítulos de libro, patentes, presentaciones en congreso, entre otros).

Sin más por el momento quedo de usted.

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICION "SALVADOR ZUBIRAN"
30 ABR 2018
DEPARTAMENTO DE NEFROLOGÍA Y METABOLISMO MINERAL

Atentamente,

Dr. Jorge Barrios Payán
Secretario de la Comisión de Investigación en Animales

c.c.p. M.V.Z. Mariela Contreras Escamilla, Jefa del DIEB

NABS/nom

INVEST. EXPERIMENTAL Y BIOTERIO
04 MAY 2018
INST. NAL. CIENCIAS MEDICAS Y NUTRUCIÓN INCMYN "S.Z."
1827 13:42

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INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN

"2015, Año del Generalísimo José María Morelos y Pavón"

México, D. F., a 10 de Julio del 2015.

Dra. Norma A. Bobadilla Sandoval
Depto. de Nefrología y Metabolismo Mineral
P r e s e n t e .

REF: CINVA-45 NMM-45-11-13-1

Estimada Dra. Bobadilla:

Habiendo analizado detalladamente el Protocolo de Investigación Experimental titulado:

"Implicación de la aldosterona en el desarrollo de enfermedad renal crónica como consecuencia de una lesión renal aguda"

Este comité ha dictaminado **aprobar la prorroga con vigencia de dos años a partir de esta fecha.** En el caso de concluir antes el protocolo, favor de enviar la carta de cierre del mismo.

Sin más por el momento quedo de usted.

Atentamente,

Dr. Jorge Barrios Payán
Comisión de Investigación en Animales

401

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN SALVADOR ZUBIRAN
INCMNSZ
22 JUL 2015 09:00
DEPARTAMENTO DE INVESTIGACIÓN EXPERIMENTAL Y BIOTERIO

c.c.p. Dr. Gerardo Gamba, Director de Investigación
M.V.Z. Mariela Contreras Escamilla, Jefa del Bioterio

INS. NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN "SALVADOR ZUBIRÁN"
22 JUL 2015
DIRECCIÓN DE INVESTIGACIÓN

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INSTITUTO NACIONAL DE
CIENCIAS MÉDICAS
Y NUTRICIÓN
SALVADOR ZUBIRÁN

"2015, Año del Generalísimo José María Morelos y Pavón"

México, D.F. a 3 de Julio del 2015

Comité de la CINVA
Presente

Estimado Comité de la CINVA:

Por este conducto me permito solicitar una prórroga de dos años del protocolo: **"IMPLICACIÓN DE LA ALDOSTERONA EN EL DESARROLLO DE ENFERMEDAD RENAL CRÓNICA COMO CONSECUENCIA DE UNA LESIÓN RENAL AGUDA",** con registro **CINVA: NMM-45-11-13-1,** la solicitud se basa que aunque ya terminamos todos los experimentos en animales e incluso hemos publicado ya un par de artículos, en este momento nos encontramos realizando los estudios moleculares e histopatológicos de otra parte del proyecto por lo que, en cuanto terminemos enviaremos el manuscrito, sin embargo no sabemos si los revisores nos pedirán más experimentos y nos gustaría mantener vigente ante la CINVA el protocolo

Agradezco la atención prestada a la presente.

Atentamente,

Dra. Norma A. Bobadilla Sandoval
Departamento de Nefrologia y Meatbolismo Mineral

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INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN

"2015, Año del Generalísimo José María Morelos y Pavón"

México, D.F. a 23 de Junio del 2015

Dra. Norma Bobadilla Sandoval
Depto. de Nefrología y
Metabolismo Mineral
Presente

Estimada Dra. Bobadilla:

Por este conducto le informo que su proyecto: **"IMPLICACIÓN DE LA ALDOSTERONA EN EL DESARROLLO DE ENFERMEDAD RENAL CRÓNICA COMO CONSECUENCIA DE UNA LESIÓN RENAL AGUDA"**, con registro **CINVA: NMM-45-11-13-1** finalizó en mayo de este año. Por lo que, le solicito de la manera más atenta me haga saber si el proyecto requerirá una prórroga. En caso afirmativo, favor de enviar a la **CINVA** el periodo de extensión que solicita y de requerir un mayor número de animales especificar y justificar como se utilizarán y los procedimientos experimentales que se llevarán a cabo con los mismos. En caso de no requerir una prórroga favor de llenar el formato de cierre del protocolo que se anexa a la presente.

Sin otro particular por el momento, quedo de usted.

Atentamente,

Dr. Jorge Barrios Payan
CINVA

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN
INCMNSZ
30 JUN 2015
DEPARTAMENTO DE INVESTIGACIÓN EXPERIMENTAL Y BIOTERIO
1:31 pm

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN
30 JUN 2015
COMITE DE INVESTIGACIÓN

c.c.p. Dr. Gerardo Gamba Ayala, Director de Investigación.
MVZ Mariela Contreras Escamilla, Jefa del Bioterio.

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INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRÁN

Abril 23, 2012

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN "SALVADOR ZUBIRÁN"
09 MAY 2012
DEPTO. DE NEFROLOGÍA Y METABOLISMO MINERAL

Dra. Norma A. Bobadilla Sandoval
Investigador en Ciencias Médicas F
Departamento de Nefrología y Matabolismo Mineral
P r e s e n t e .

Con referencia al proyecto de investigación: **"Implicación de la aldosterona en la enfermedad renal crónica (ERC) como consecuencia de la lesión renal aguda".**

Registro CINVA: 45

Clave: NMM-45-11-13-1

La Comisión de Investigación en Animales (CINVA), revisó su respuesta a las observaciones emitidas por esta Comisión y se decidió **APROBARLO** para su desarrollo.

A t e n t a m e n t e

MVZ., M. Sc, Cert.L.A.M. Rafael Hernández González
Coordinador de la Comisión de Investigación en Animales

ccp. Dr. Rubén Lisker Y.- Director de Investigación
MVZ., M.en C. Octavio Villanueva Sánchez .Secretario de la Comisión de Investigación en Animales
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Dra. Nimbe Torres y Torres.- Comisión de Investigación en Animales

INNSZ

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRÁN

México D.F. a 11 de Abril de 2012

MVZ. M.Sc. Rafael Hernández González
Coordinador de la Comisión de
Investigación en Animales (CINVA)

Estimado Dr. Hernández:

Me permito enviarle a usted el proyecto Registro: **CINVA: 45**, CLAVE: NMM-45-11-13-1, en el que se realizaron las correcciones indicadas por la Comisión de Investigación en Animales (CINVA), desafortunadamente no las imprimimos cuando aparecieron en el sistema y actualmente no tenemos acceso a las mismas.

Esperando contar con la anuencia de la comisión quedo de usted.

Atentamente

Dra. Norma A. Bobadilla Sandoval
Investigador en Ciencias Medicas F
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INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRAN

Dirección de Investigación

FORMA ÚNICA PARA REGISTRO DE PROYECTOS

No invada las zonas sombreadas

CLAVE: NMM - 45 - 11 / 13 - 1

FECHA DE RECEPCIÓN : 2011-01-20

TITULO: Implicación de la aldosterona en el desarrollo de enfermedad renal crónica como consecuencia de una lesión renal aguda

INVESTIGADOR RESPONSABLE: NORMA BOBADILLA SANDOVAL

DEPARTAMENTO O SERVICIO: NEFROLOGIA Y METABOLISMO MINERAL

TIPO DE INVESTIGACIÓN:

	1. Investigación Clinica
X	*2. Investigación Experimental*
	3. Investigación Documental
	4. Desarrollo Tecnológico
	5. Investigación Epidemiológica
	6. Otros

PATROCINADORES:

	Cantidad:
0	0
0	*TOTAL*
0	0

PERIODO DE UTILIZACIÓN DE LOS RECURSOS: *de mes:* 01 *año:* 2011 *a mes:* 12 *año* 2013

FORMA EN LA QUE SE RECIBIRÁN LOS FONDOS:

Primer año:	0	0	0	0
Segundo año:	0	0	0	0
Tercer año:	0	0	0	0
Cuarto año:	0	0	0	0
Quinto año:	0	0	0	0

COSTOS TOTALES DE LA INVESTIGACIÓN

1. Personal	0
2. Equipos:	0
3. Materiales	0
4. Animales	0
5. Estudios	0
6. Viáticos:	0
7. Pubs. apoyos	0
8. Suscripcion	0
9. Varios	0
10. Fondo de Apoyo	0
TOTAL:	0

INSTITUCIONES PARTICIPANTES

FIRMAS

El presente formulario pretende facilitar la evaluación de su proyecto e identificar las necesidades de animales de laboratorio, equipo y manejo para su proyecto de investigación. Para el llenado de los cuadros consulte la información que describe cada sección de los mismos:

Fecha de inicio del estudio: 31 de enero 2010

Fecha de terminación del estudio: 15 DIC 2013

Indique con el inciso correspondiente las características de los animales, condiciones de alojamiento y maniobras experimentales que requiere el proyecto:

1. Especie	2. Raza, cepa	3. Condición microbiol.	4. No. H	Total M	5. Distribución	6. Alojamiento	7. Densidad	8. Nivel biosegur.	9. Nivel de afectación	10. Destino	11. Eutanasia
Rata	Wistar	A	---------	480	5 por semana	A, C	Animal por caja	Nivel I	Categoría D	C	Sobredosis Anestesia Intravenosa

1. Nombre genérico o especie: escriba el nombre común o científico de los animales que empleará en su estudio.

2. Raza o cepa o tipo genético: escriba la nomenclatura que mejor describe las características genéticas del animal que necesita (ej: ratón: BALB/c, C57BL6, Cd1, un/un, Rata: wistar, Fischer 344, sprague-dawley, NHI, Conejo: nueva Zelanda albino, Hámster: dorado)

3. Tipo o condición microbiológica: A) convencional: animal con flora microbiológica desconocida, sin signos aparentes de enfermedad, B) SPF: (specific pathogen free) libre de patógenos específicos (indicar tipo de patógenos indeseables ej: virus, bacterias, hongos, parásitos), C) Otro: especifique.

4. Numero total: indique el número de animales que utilizará en el estudio, H: hembras, M: machos, incluyendo grupos piloto. En cao de utilizar animales de un sólo sexo favor de invalidar la columna correspondiente.

5. Distribución: indique la cantidad y la frecuencia en que se requiere que se le entreguen los animales ej: todos en una entrega, 10 cada semana, al mes, bimestre, trimestre, semestre, etc.

6. Alojamiento: indique con letra el tipo que corresponda:
   A) Caja de policarbonato de piso sólido
   B) Jaula con piso de malla o rejilla
   C) Jaula metabólica
   D) Microaislador
   E) Caja de policarbonato de piso sólido con filtro
   F) Perrera
   G) Corral
   H) Corraleta metabólica
   I) Pecera

7. Densidad poblacional: indique como alojará a los animales ej: un animal por caja, parejas, 3, 5, etc. Consultar la Norma Oficial Mexicana NOM-062-ZOO-1999, Especificaciones técnicas para la producción cuidado y uso de los animales de laboratorio. Publicada por la SAGARPA en el Diario Oficial el miércoles 22 de agosto del 2001.

8. Nivel de bioseguridad: Deberá indicar el nivel de riesgo biológico que existe para el personal que maneja a los animales o sus desechos, tanto para los investigadores, alumnos y técnicos del bioterio.
   Nivel I) Trabajo con agentes químicos, físicos o biológicos que no producen enfermedad y no son un riesgo para la salud de personas sanas y el medio ambiente
   Nivel II) Trabajo con agentes químicos, físicos o biológicos que tienen un peligro potencial bajo o moderado para la salud del personal y del medio ambiente (ej: Salmonelosis, Toxoplasmosis, Hepatitis B)
   Nivel III) Trabajo con agentes químicos, físicos o biológicos que tienen un peligro potencial alto para la salud humana y animal o pueden producir la muerte cuando se inhalan (ej: Tuberculosis, *Coxiella Burnetti*)
   Nivel IV) Trabajo con agentes químicos, físicos o biológicos exóticos transmisibles por aerosoles y mortales para seres humanos y animales (ej: virus ébola, virus hanta)

9. Nivel de afectación de los animales indique el nivel de invasividad y el grado de dolor que sentirá el animal durante los procedimientos experimentales o manipulación:
   Categoría A) Experimentos con invetebrados, huevos, protozoarios, organismos unicelulares. Uso de metazoarios, cultivo de tejidos u órganos obtenidos después de la muerte del animal.
   Categoría B) Experimentos que causen molestia o estrés mínimo (inyección no dolorosa, restricción de movimiento, marcado o aretado de las orejas)
   Categoría C) Experimentos que causan estrés menor o dolor de corta duración, realizados con analgensia o anestesia (colocación de cánula, biopsia, cirugía menor)
   Categoría D) Experimentos que causan estrés o dolor moderado a severo controlado con anestesia (procedimientos quirúrgicos mayores)

10. Destino final: indicar el destino final de los animales al termino de los experimentos:
    A) Vivo sin cirugía
    B) Vivo post-cirugía
    C) Cirugía terminal (no despierta de la anestesia)
    D) Eutanasia

11. Eutanasia indique el método empleado para dar muerte al animal. Consultar la Norma Oficial Mexicana NOM-062-ZOO-1999, Especificaciones técnicas para la producción cuidado y uso de los animales de laboratorio. Publicada por la SAGARPA en el Diario Oficial el miércoles 22 de agosto del 2001, capitulo 9, eutanasia.

En el siguiente cuadro marque con la clave que se indica en el nivel de habilidad y experiencia de usted y su personal para realizar las maniobras experimentales mencionadas en la columna de la izquierda.

Clave:

A) Entrenado, hábil y con experiencia.
B) Será entrenado y supervisado por el I.P
C) Requiere instrucción, entrenamiento y supervisión por el personal del D.I.E.B.

En caso de que las maniobras experimentales sean realizadas por el personal del bioterio se marcara con una X el espacio correspondiente en la columna D.I.E.B.

Investigador o personal que trabajara directamente con los animales (investigadores asociados, alumnos, tesistas, etc)

MANIOBRA	I.P	1	2	3	4	D.I.E.B.
Inmovilización	A	A	B			
Anestesia	A	A	B			
Medicación Enteral	-------	-------	-------			
Medicación Parenteral	-------	-------	-------			
Toma de sangre	A	A	B			
Otras Muestras*	-------	-------	-------			
Cirugía	A	A	B			
Eutanasia	A	A	B			
Otras **	-------	-------	-------			

* Especificar de qué, cantidad y frecuencia de muestreo: _____

_____

** Especificar: _____

Dra. Norma A. Bobadilla Sandoval

Implicación de la aldosterona en la enfermedad renal crónica (ERC) como consecuencia de la lesión renal aguda

Antecedentes:

La aldosterona es una hormona mineralocorticoide cuya acción clásica es el mantenimiento del volumen extracelular a través de aumentar la reabsorción de sodio y promover la excreción de potasio. La mayoría de las acciones conocidas de la aldosterona, tanto en células epiteliales como no epiteliales son mediadas a través de la activación de los receptores mineralocorticoides, los cuales son capaces de regular la expresión y trascripción de diversos genes (1). Los receptores mineralocorticoides se encuentran en el túbulo distal y colector pero también se han localizado en otros tejidos como corazón, cerebro y endotelio vascular. Esta localización junto con la evidencia reciente en humanos y en animales que muestra que la aldosterona juega un papel importante en la fisiopatología de las enfermedades cardiovasculares y renales ha modificado en forma importante el conocimiento de las acciones de la aldosterona (2,3).

El estudio pionero que demostró que la aldosterona participa en la fisiopatología de la enfermedad renal, fue realizado en 1996 por el grupo de Thomas Hostetter (4), quienes al utilizar el modelo clásico de progresión de daño renal, el de la nefrectomía 5/6 en la rata, demostraron que el uso combinado de un inhibidor de la enzima convertidora de angiotensina (enalapril) y un bloqueador de los receptores AT1 de angiotensina (losartan), reducía, como era de esperarse, la proteinuria y el porcentaje de glomeruloesclerosis. Este efecto renoprotector del enalapril y losartan, no era observado cuando se restablecían los niveles de aldosterona al mismo nivel que el grupo no tratado. Estos resultados probaron por primera vez, que el daño renal en este modelo era mediado en gran parte por la aldosterona.

En la práctica médica, tres estudios, dos piloto y un doble ciego controlado con placebo evidenciaron que el tratamiento con espironolactona reduce de forma importante la excreción urinaria de proteínas en pacientes con ERC, en estos estudios se observó además, una correlación significativa entre los niveles de proteinuria y los niveles de aldosterona, es decir, los pacientes que presentaban mayor excreción urinaria de proteínas eran los que tenían mayor aldosterona circulante (5,6). Estos estudios clínicos indican que el bloqueo de los receptores de mineralocorticoides reduce el daño renal e incluso ofrece un efecto protector adicional sobre el uso de inhibidores de la enzima convertidora de angiotensina y antagonistas de los receptores AT1 en los pacientes con ERC.

Estudios recientes de nuestro laboratorio analizaron el efecto de la administración de espironolactona en ratas con nefrotoxicidad crónica por CsA. La administración de CsA durante 21 días indujo daño estructural renal caracterizado por arteriolopatía y fibrosis tubulointersticial, asociado a un aumento en las concentraciones de RNAm del TGF- β, fibronectina y colágena I y IV. Por su parte, las ratas que recibieron simultáneamente CsA y espironolactona presentaron una reducción significativa de arteriolopatía así como de fibrosis tubulointersticial. Este efecto renoprotector se relacionó con la prevención de la sobre-expresión del TGF- β y de proteínas de la matriz extracelular (7). De forma simultánea la administración de espironolactona inhibió por completo la reducción en la depuración de la creatinina, lo que sugiere que la aldosterona es un mediador importante tanto del daño funcional como del estructural en este modelo de nefropatía y que está implicada en la regulación del tono vascular renal en este modelo.

Para investigar si la aldosterona esta involucrada en la regulación del tono vascular renal, estudiamos el efecto de la espironolactona sobre el flujo sanguíneo renal y la tasa de filtración glomerular en ratas tratadas con CsA durante 7 días para producir un modelo agudo y reversible con CsA el cual se caracteriza por vasoconstricción potente (8). En este estudio observamos que la administración de espironolactona inhibió por completo la caída en la tasa de filtración glomerular inducida por la CsA, logrando el reestablecimiento del flujo sanguíneo renal.

Finalmente completamos el abordaje de este efecto renoprotector al estudiar el efecto de la espironolactona en ratas tratadas previamente con CsA por 18 días y con nefrotoxicidad crónica documentada. Se administró espironolactona junto con CsA por 18 días más y se comparó con un grupo que sólo recibió CsA durante un periodo similar (9). Aunque no se logró revertir la disfunción renal ya establecida, si se evitó un mayor deterioro de la función renal. Estructuralmente, la espironolactona

brindó una protección renal significativa asociada a la reducción del engrosamiento de las arteriolas, apoptosis y fibrosis tubulointersticial.

En conjunto, nuestros resultados sugirieron fuertemente, que la aldosterona modula el tono de la vasculatura renal y que en la nefrotoxicidad por CsA produce vasoconstricción renal y contribuye al daño por este inmunosupresor. Si esto es cierto, entonces el bloqueo de los receptores de mineralocorticoides podría ser un agente protector contra el daño renal inducido por isquemia/reperfusión donde la vasoconstricción renal tiene un papel preponderante.

El daño renal por isquemia-reperfusión (I/R) es la mayor causa de LRA en riñones nativos y transplantados (10). La LRA es un síndrome que se desarrolla después de una caída transitoria en flujo sanguíneo renal, produciéndose un incremento abrupto en los niveles de creatinina sérica, como resultado del daño funcional y/o estructural causado por el riñón. Para investigar el papel de la aldosterona sobre la vasoconstricción renal observada en la LRA, utilizamos el modelo de daño renal inducido por isquemia/reperfusión. Este modelo se caracteriza por la elevación de los niveles de aldosterona plasmática así como una caída del flujo sanguíneo renal. Las ratas sometidas a isquemia desarrollaron disfunción renal, que se caracterizó por un aumento en los niveles de creatinina sérica, como consecuencia de la reducción de la tasa de filtración glomerular. De manera interesante, en los tres grupos que recibieron el tratamiento profiláctico con espironolactona, se previno completamente la elevación de la creatinina sérica, debido al restablecimiento de la función renal a valores normales(11).

Estos resultados muestran que la aldosterona participa en el desarrollo de la LRA y que su bloqueó muestra ser una herramienta útil para el tratamiento de este padecimiento. Sin embargo, aun quedaba la posibilidad de que la espironolactona ejerciera sus efectos por acciones no especificas, es decir, que la renoprotección no fuera mediada a través del bloqueo de los receptores a mineralocorticoides, sino a través de un mecanismo indirecto. Para resolver esta interrogante, diseñamos un estudio para evaluar si la ausencia de aldosterona tiene efectos similares a los efectos de la espironolactona en prevenir el daño renal inducido por I/R (12). Por lo tanto, en un estudio publicado por nuestro grupo recientemente evaluamos si la adrenalectomía, maniobra en la que los niveles séricos de aldosterona son prácticamente nulos, se previenen las lesiones inducidas por un fenómeno de I/R. Como era de esperarse, la I/R produjo disfunción renal y necrosis tubular severa que se asoció con un aumento significativo de los marcadores de daño tubular. En contraste, la hipoperfusión e hiperfiltración, así como la necrosis tubular aguda inducida por I/R, no se observó en los animales que fueron adrenalectomizados antes de inducir I/R.

En conjunto todos nuestros estudios previos sugieren fuertemente que: 1) la aldosterona juega un papel clave en mediar el daño renal por I/R, 2) que el efecto benéfico de la espironolactona es debido a su habilidad de bloquear a los receptores a mineralocorticoides y 3) que el antagonismo de los receptores a mineralocorticoides puede ser utilizado como una estrategia para prevenir la LRA.

Por otra parte, estudios recientes han mostrado que el haber padecido de LRA es un factor de riesgo para desarrollar enfermedad renal crónica (ERC), sin embargo, poco se conoce acerca de los mecanismos involucrados (13,14,15). Una consecuencia importante de la LRA es que puede incrementar la progresión del daño renal crónico preexistente y el desarrollo de ERC terminal (16). Por lo que, resulta indispensable el estudio de los mecanismos que desencadena la LRA para afectar la función y estructura a largo plazo y encontrar maniobras farmacológicas que eviten el desarrollo de ERC.

Objetivo general

1. Establecer si el bloqueo de los receptores a mineralocorticoides antes o después de inducir una LRA severa puede evitar o prevenir la progresión a enfermedad renal crónica.

Objetivos particulares

1. Desarrollar y caracterizar un modelo de LRA que conlleve al desarrollo de ERC.
2. Evaluar los posibles mecanismos responsables por lo que un periodo de isquemia pueden llevar al desarrollo de ERC. Entre ellos, procesos fibróticos y de trans-diferenciación epitelio-mesénquima, alteraciones en la proliferación celular y activación de la respuesta inflamatoria.

3. Determinar si la administración antes o después de inducir lesión renal aguda puede prevenir el desarrollo de alteraciones funcionales y estructurales observadas en la ERC.
4. Evaluar los posibles mecanismos de la renoprotección conferida por espironolactona en la prevención de la progresión a ERC.

Material y Métodos

Inducción de los modelos experimentales:

Modelo de Isquemia/reperfusión (I/R): Las ratas se anestesiarán y se interrumpirá el flujo a los riñones mediante la colocación de un clip en cada arteria renal durante 45 min. Los animales se suturarán y se dejarán evolucionar por 180 y 270 días hasta su sacrificio. La espironolactona se administrará intra-gástricamente con una dosis de 20 mg/kg en dos formas diferentes: profilácticamente y después de inducir I/R. Para el protocolo profiláctico, la espironolactona se administrará 72 h antes de inducir I/R y para el segundo protocolo, 3 h posteriores a la inducción de la I/R.

Grupos a incluir y Seguimiento de los animales: Se incluirán cinco grupos experimentales cada uno constituido por 24 animales, la mitad de las ratas se sacrificarán a los 180 días y la otra mitad a los 270 días

1) Ratas a las que se les practicará una cirugía falsa y servirán como el grupo control (Sham).

2) Ratas a las que se les administrará esprironolactona 72 h antes de la cirugía falsa (Sham+Sp)

3) Ratas que se someterán a isquemia bilateral de 45 min y reperfusión posterior (I/R)

4) Ratas a las que se les administrara espironolactona 72 h antes de inducir I/R (Sp+I/R)

5) Ratas a las que se les inducirá I/R y que recibirán espironolactona 3 horas después de la isquemia.

Los animales se mantendrán durante 180 o 270 días en un ciclo de luz-obscuridad 12:12 y con acceso libre al alimento y agua. Cada 30 días se colocarán en jaulas metabólicas para recolección de orina de 24 h.

1. **Mediciones fisiológicas:** Se registrara el peso corporal, consumo de alimento y agua a lo largo del estudio, todos los animales se colocaran en jaulas metabólicas para la recolección de orina de 24 horas al inicio del protocolo y cada 30 días, cuantificando el volumen urinario, también se tomará una muestra de sangre para medir creatinina, osmolaridad, hematocrito, niveles séricos de, aldosterona, angiotensina I y renina, también se determinará la proteinuria y se registrara la presión arterial de los animales. Se determinara la tasa de filtración glomerular, flujo sanguíneo renal, electrolitos, se llevaran a cabo estudios histopatológicos y moleculares, cuantificación de lipoperoxidación de tejido renal.
   Filtración glomerular: Las ratas se anestesiaran con pentobarbital sódico (30 mg/kg de peso corporal i.p.). La preparación quirúrgica se realizará por medio de microcirugía de la siguiente forma: se colocará a la rata en la mesa termoregulada a 37 °C, se cateterizaran la tráquea con un tubo de polietileno PE-240, las venas yugulares, la arteria femoral con tubo de polietileno PE-50, y la vejiga con tubo de polietileno PE-90. Durante todo el experimento se registrará la presión arterial media en un polígrafo Grass. Las ratas se mantendrán en condiciones de euvolemia por la infusión de 10 ml/kg de plasma durante la cirugía seguida de una infusión de de azúcar baja en calorías (BC) al 5%[27] después de un periodo de equilibrio de 45 min, se recolectará la orina durante 30 minutos, tomándose muestras de sangre de la arteria femoral al inicio y al final de la

recolección. El volumen de sangre será reemplazado con sangre de una rata donadora por la vena yugular. La concentración de azúcar BC en plasma y orina se determinará por el método de Davidson [28].

Flujo Sanguíneo Renal (FSR): Una vez realizada la cirugía y que el animal se encuentre en condiciones de euvolemia, se disecará la arteria renal izquierda y se colocará una sonda conectada al flujometro Transonic System en donde se registrará en forma continua el FSR.

Sodio y Potasio: El sodio y el potasio se determinarán en suero y orina por el método ión-selectivo utilizando el aparato analizador de electrolitos Nova-4.

Estudios Histopatológicos: El riñón izquierdo será perfundido al finalizar el estudio a través de la arteria femoral con una solución de fosfatos y fijado con una solución de formol al 10 %, posteriormente será embebido en parafina y se realizaran cortes histológicos, se realizara una tinción de PAS y rojo de sirio, para determinar la presencia de arteriolopatía y el grado de fibrosis túbulo intersticial mediante microscopia de luz respectivamente. Además se cuantificara el grado de apoptosis mediante la técnica de túnel. Para estudiar si la proliferación celular se encuentra afectada se realizarán inmunotinciones para PCNA en los cortes de tejido renal.

Estudios Moleculares: Se extraerá el riñón derecho y se separa microscópicamente en corteza y médula renal y se congelará en nitrógeno líquido y se almacenará a -80°C hasta su procesamiento.

Extracción de RNA total: Se extraerá el RNA total de la corteza y medula renal por homogenización con tripiure. Una vez aislado el RNA, se determinará pureza y concentración mediante espectrofotometría y mediante un gel de agarosa al 1% se verificará la integridad del mismo. ***RT-PCR en tiempo real:*** Se llevará a cabo la transcripción reversa (TR)[29,30, 31] a partir del RNA total y posteriormente, la amplificación del DNAc mediante PCR en tiempo real para los genes TGF-beta, alfa-SMA, Hsp72, HIF1-alfa, Twist, Cinasa Rho, Kim-1, procaspasa 3, Bcl 2, Brcal, enzimas antioxidantes (glutatión peroxidasa, catalasa y superoxido dismutasa) y 18S, este ultimo como gen control mediante RT-PCR en tiempo real, utilizando oligonucleótidos específicos para cada gen y una sonda marcada con un fluoróforo para detectar la amplificación por PCR en tiempo real. Se calcularan los niveles relativos de expresión para cada uno de los genes antes mencionados en relación al gen control.

Análisis de Western Blot: Las proteínas serán extraídas por homogenización corteza y medula renal en 4 volúmenes de buffer de lisis (225mM manitol, 75mM de sacarosa, 0.1mM de EDTA, 0.5 mM de MOPS, 5 mM de benzamidina y 5mM de DTT), los homogenizados serán centrifugados a 4000 g por 8 min, se retirarán los restos celulares y se determinará la concentración de proteínas por el método de Lowry. Se cargaran 50mg de proteínas en un gel SDS- PAGE al 7.5%. Posteriormente, serán transferidas a una membrana PVDF, y se incubarán con anticuerpos específicos para E-caderina, alfa-SMA, Fibronectina, Colágena I y IV, TGF-β, Smad 2 y 3, así como el estado de fosforilación de Smad, la actina se utilizará como proteína control. Para estudiar la posible participación de las MAP cinasas y de la vía del NfkappaB, se determinaran los niveles de proteína de ERK 1 y de su estado de fosforilación así como del factor de transcripción NFkappaB y su forma fosforilada (activa). La cantidad de proteína se detectará mediante un ensayo de quimiolumnicencia por autorradiografía de las bandas, y se cuantificarán por análisis densitométrico.

Evaluación de los niveles de lipoperoxidación: En muestras de homogenado de tejido renal se determinaran los niveles de malondialdehido como marcador de la lipoperoxidación de membranas celulares, a través de la técnica de TBARS.

2. **Alimentación:** NO
3. **Agua:** NO
4. **Maniobras conductuales:** NO
5. **Modificaciones Ambientales:** Se emplearan cajas de policarbonato de piso sólido con camas de aserrín para cada una de las ratas, para su estancia en el bioterio, además será necesario el uso de jaulas metabólicas al inicio del tratamiento y 24 horas antes del sacrificio, para recolección de orina de 24 horas.
6. **Restricción física y ejercicio:** NO
7. **Inmunización:** NO

8. **Administración de medicamentos:** Se empleará anestésicos: como éter para recolectar muestras de sangre al inicio del tratamiento, ketamina: xilasina (dosis: 50/10) al momento de realizar los experimentos de determinación de tasa de filtración glomerular.
9. **Inoculación de agentes biológicos**: NO
10. **Uso de sustancias peligrosas**: NO
11. **Radiaciones**: NO
12. **Trauma**: NO
13. **Cirugía**: 10 ratas de cada grupo serán anestesiadas con ketamina/Xilasina (50/10 mg/Kg de peso corporal i.p.). La preparación quirúrgica se realizará por medio de microcirugía de la siguiente forma: se colocará a la rata en la mesa termoregulada a 37 °C, se cateterizaran la tráquea con un tubo de polietileno PE-240, las venas yugulares, la arteria femoral con tubo de
14. polietileno PE-50, y la vejiga con tubo de polietileno PE-90. Durante todo el experimento se registrará la presión arterial media en un polígrafo Grass. Las ratas se mantendrán en condiciones de euvolemia por la infusión de 10 ml/kg de plasma durante la cirugía seguida de una infusión de azúcar BC 5% y después de un periodo de equilibrio de 45 min, se recolectará la orina durante 30 minutos, tomándose muestras de sangre de la arteria femoral al inicio y al final de la recolección. El volumen de sangre será reemplazado con sangre de una rata donadora por la vena yugular.

    Otras106 ratas de cada grupo serán anestesiadas nuevamente con ketamina/Xilasina (50/10 mg/Kg de peso corporal i.p.). Se colocarán en una mesa termoregulada a 37 °C, se realizara una incisión media en el abdomen, posteriormente se removerá el riñón derecho, separándolo macroscópicamente en corteza y médula renal, se congelará en nitrógeno líquido y se almacenará a -80°C hasta su procesamiento para los estudios moleculares. Finalmente las ratas restantes de cada grupo se anestesiaran con el mismo analgésico y relajante muscular antes mencionado (50/10 mg/Kg de peso corporal i.p.). La preparación quirúrgica se realizará por medio de microcirugía de la siguiente forma: se colocará a la rata en la mesa termoregulada a 37 °C, se cateterizaran la tráquea con un tubo de polietileno PE-240 y una arteria femoral con tubo de polietileno PE-50, posteriormente serán perfundidos los riñones a través de la arteria femoral con una solución de fosfatos y fijados con una solución de formol al 10 %, posteriormente serán embebidos en parafina y se realizaran cortes histológicos, para determinar los cambios histológicos característicos del modelo experimental.

    Obtención de muestras: Se recolectara orina de 24 horas mediante la colocación de los animales en jaulas metabólicas, Volumen: 4ml, Conservación: -20°C, Frecuencia: Basal y cada 30 días, condiciones previas: ninguna, Se recolectara muestra de sangre al inicio del tratamiento a través de seno ocular usando capilar, al finalizar el estudio a través de la arteria femoral, Volumen: 1ml, Conservación: -20°C, Frecuencia: Basal y al finalizar los 36 días, condiciones previas: ninguna.

15. **Obtención de tejidos**: Se obtendrán los riñones a través de escisión quirúrgica, bajo anestesia.
16. **Eutanasia**: se llevara a cabo a través de una sobredosis de anestesia por vía intravenosa.
17. **Disposición de cadáveres**: será determinado de acuerdo con lo establecido en la NOM-087-ECOL-1995

Análisis estadístico

Los resultados obtenidos se analizarán por ANOVA de una vía y las diferencias entre grupos se determinarán con la prueba de Student-Neumann-Kuels. Los datos se presentarán como el promedio ± el error estándar.

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ANEXOS AL PROYECTO CON TÍTULO:

Implicación de la aldosterona en el desarrollo de enfermedad renal crónica como consecuención de una lesión renal aguda

Hemos recibido:

Forma única de Registro, Proyecto completo y los siguientes anexos:

Implicación de la aldosterona en la enfermedad renal crónica (ERC) como consecuencia de la lesión renal aguda

Antecedentes:

La aldosterona es una hormona mineralocorticoide cuya acción clásica es el mantenimiento del volumen extracelular a través de aumentar la reabsorción de sodio y promover la excreción de potasio. La mayoría de las acciones conocidas de la aldosterona, tanto en células epiteliales como no epiteliales son mediadas a través de la activación de los receptores mineralocorticoides, los cuales son capaces de regular la expresión y trascripción de diversos genes (1). Los receptores mineralocorticoides se encuentran en el túbulo distal y colector pero también se han localizado en otros tejidos como corazón, cerebro y endotelio vascular. Esta localización junto con la evidencia reciente en humanos y en animales que muestra que la aldosterona juega un papel importante en la fisiopatología de las enfermedades cardiovasculares y renales ha modificado en forma importante el conocimiento de las acciones de la aldosterona (2,3).

El estudio pionero que demostró que la aldosterona participa en la fisiopatología de la enfermedad renal, fue realizado en 1996 por el grupo de Thomas Hostetter (4), quienes al utilizar el modelo clásico de progresión de daño renal, el de la nefrectomía 5/6 en la rata, demostraron que el uso combinado de un inhibidor de la enzima convertidora de angiotensina (enalapril) y un bloqueador de los receptores AT1 de angiotensina (losartan), reducía, como era de esperarse, la proteinuria y el porcentaje de glomeruloesclerosis. Este efecto renoprotector del enalapril y losartan, no era observado cuando se restablecían los niveles de aldosterona al mismo nivel que el grupo no tratado. Estos resultados probaron por primera vez, que el daño renal en este modelo era mediado en gran parte por la aldosterona.

En la práctica médica, tres estudios, dos piloto y un doble ciego controlado con placebo evidenciaron que el tratamiento con espironolactona reduce de forma importante la excreción urinaria de proteínas en pacientes con ERC, en estos estudios se observó además, una correlación significativa entre los niveles de proteinuria y los niveles de aldosterona, es decir, los pacientes que presentaban mayor excreción urinaria de proteínas eran los que tenían mayor aldosterona circulante (5,6). Estos estudios clínicos indican que el bloqueo de los receptores de mineralocorticoides reduce el daño renal e incluso ofrece un efecto protector adicional sobre el uso de inhibidores de la enzima convertidora de angiotensina y antagonistas de los receptores AT1 en los pacientes con ERC.

Estudios recientes de nuestro laboratorio analizaron el efecto de la administración de espironolactona en ratas con nefrotoxicidad crónica por CsA. La administración de CsA durante 21 días indujo daño estructural renal caracterizado por arteriolopatía y fibrosis tubulointersticial, asociado a un aumento en las concentraciones de RNAm del TGF- β, fibronectina y colágena I y IV. Por su parte, las ratas que recibieron simultáneamente CsA y espironolactona presentaron una reducción significativa de arteriolopatía así como de fibrosis tubulointersticial. Este efecto renoprotector se relacionó con la prevención de la sobre-expresión del TGF- β y de proteínas de la matriz extracelular (7). De forma simultánea la administración de espironolactona inhibió por completo la reducción en la depuración de la creatinina, lo que sugiere que la aldosterona es un mediador importante tanto del daño funcional como del estructural en este modelo de nefropatía y que está implicada en la regulación del tono vascular renal en este modelo.

Para investigar si la aldosterona esta involucrada en la regulación del tono vascular renal, estudiamos el efecto de la espironolactona sobre el flujo sanguíneo renal y la tasa de filtración glomerular en ratas tratadas con CsA durante 7 días para producir un modelo agudo y reversible con CsA el cual se caracteriza por vasoconstricción potente (8). En este estudio observamos que la administración de espironolactona inhibió por completo la caída en la tasa de filtración glomerular inducida por la CsA, logrando el reestablecimiento del flujo sanguíneo renal.

Finalmente completamos el abordaje de este efecto renoprotector al estudiar el efecto de la espironolactona en ratas tratadas previamente con CsA por 18 días y con nefrotoxicidad crónica documentada. Se administró espironolactona junto con CsA por 18 días más y se comparó con un grupo que sólo recibió CsA durante un periodo similar (9). Aunque no se logró revertir la disfunción renal ya establecida, si se evitó un mayor deterioro de la función renal. Estructuralmente, la espironolactona

brindó una protección renal significativa asociada a la reducción del engrosamiento de las arteriolas, apoptosis y fibrosis tubulointersticial.

En conjunto, nuestros resultados sugirieron fuertemente, que la aldosterona modula el tono de la vasculatura renal y que en la nefrotoxicidad por CsA produce vasoconstricción renal y contribuye al daño por este inmunosupresor. Si esto es cierto, entonces el bloqueo de los receptores de mineralocorticoides podría ser un agente protector contra el daño renal inducido por isquemia/reperfusión donde la vasoconstricción renal tiene un papel preponderante.

El daño renal por isquemia-reperfusión (I/R) es la mayor causa de LRA en riñones nativos y transplantados (10). La LRA es un síndrome que se desarrolla después de una caída transitoria en flujo sanguíneo renal, produciéndose un incremento abrupto en los niveles de creatinina sérica, como resultado del daño funcional y/o estructural causado por el riñón. Para investigar el papel de la aldosterona sobre la vasoconstricción renal observada en la LRA, utilizamos el modelo de daño renal inducido por isquemia/reperfusión. Este modelo se caracteriza por la elevación de los niveles de aldosterona plasmática así como una caída del flujo sanguíneo renal. Las ratas sometidas a isquemia desarrollaron disfunción renal, que se caracterizó por un aumento en los niveles de creatinina sérica, como consecuencia de la reducción de la tasa de filtración glomerular. De manera interesante, en los tres grupos que recibieron el tratamiento profiláctico con espironolactona, se previno completamente la elevación de la creatinina sérica, debido al restablecimiento de la función renal a valores normales(11).

Estos resultados muestran que la aldosterona participa en el desarrollo de la LRA y que su bloqueó muestra ser una herramienta útil para el tratamiento de este padecimiento. Sin embargo, aun quedaba la posibilidad de que la espironolactona ejerciera sus efectos por acciones no especificas, es decir, que la renoprotección no fuera mediada a través del bloqueo de los receptores a mineralocorticoides, sino a través de un mecanismo indirecto. Para resolver esta interrogante, diseñamos un estudio para evaluar si la ausencia de aldosterona tiene efectos similares a los efectos de la espironolactona en prevenir el daño renal inducido por I/R (12). Por lo tanto, en un estudio publicado por nuestro grupo recientemente evaluamos si la adrenalectomía, maniobra en la que los niveles séricos de aldosterona son prácticamente nulos, se previenen las lesiones inducidas por un fenómeno de I/R. Como era de esperarse, la I/R produjo disfunción renal y necrosis tubular severa que se asoció con un aumento significativo de los marcadores de daño tubular. En contraste, la hipoperfusión e hiperfiltración, así como la necrosis tubular aguda inducida por I/R, no se observó en los animales que fueron adrenalectomizados antes de inducir I/R.

En conjunto todos nuestros estudios previos sugieren fuertemente que: 1) la aldosterona juega un papel clave en mediar el daño renal por I/R, 2) que el efecto benéfico de la espironolactona es debido a su habilidad de bloquear a los receptores a mineralocorticoides y 3) que el antagonismo de los receptores a mineralocorticoides puede ser utilizado como una estrategia para prevenir la LRA.

Por otra parte, estudios recientes han mostrado que el haber padecido de LRA es un factor de riesgo para desarrollar enfermedad renal crónica (ERC), sin embargo, poco se conoce acerca de los mecanismos involucrados (13,14,15). Una consecuencia importante de la LRA es que puede incrementar la progresión del daño renal crónico preexistente y el desarrollo de ERC terminal (16). Por lo que, resulta indispensable el estudio de los mecanismos que desencadena la LRA para afectar la función y estructura a largo plazo y encontrar maniobras farmacológicas que eviten el desarrollo de ERC.

Objetivo general

1. Establecer si el bloqueo de los receptores a mineralocorticoides antes o después de inducir una LRA severa puede evitar o prevenir la progresión a enfermedad renal crónica.

Objetivos particulares

1. Desarrollar y caracterizar un modelo de LRA que conlleve al desarrollo de ERC.
2. Evaluar los posibles mecanismos responsables por lo que un periodo de isquemia pueden llevar al desarrollo de ERC. Entre ellos, procesos fibróticos y de trans-diferenciación epitelio-mesénquima, alteraciones en la proliferación celular y activación de la respuesta inflamatoria.

3. Determinar si la administración antes o después de inducir lesión renal aguda puede prevenir el desarrollo de alteraciones funcionales y estructurales observadas en la ERC.
4. Evaluar los posibles mecanismos de la renoprotección conferida por espironolactona en la prevención de la progresión a ERC.

Material y Métodos

Inducción de los modelos experimentales:

Modelo de Isquemia/reperfusión (I/R): Las ratas se anestesiarán y se interrumpirá el flujo a los riñones mediante la colocación de un clip en cada arteria renal durante 45 min. Los animales se suturarán y se dejarán evolucionar por 180 y 270 días hasta su sacrificio. La espironolactona se administrará intra-gástricamente con una dosis de 20 mg/kg en dos formas diferentes: profilácticamente y después de inducir I/R. Para el protocolo profiláctico, la espironolactona se administrará 72 h antes de inducir I/R y para el segundo protocolo, 3 h posteriores a la inducción de la I/R.

Grupos a incluir y Seguimiento de los animales: Se incluirán cinco grupos experimentales cada uno constituido por 24 animales, la mitad de las ratas se sacrificarán a los 180 días y la otra mitad a los 270 días

1) Ratas a las que se les practicará una cirugía falsa y servirán como el grupo control (Sham).

2) Ratas a las que se les administrará esprironolactona 72 h antes de la cirugía falsa (Sham+Sp)

3) Ratas que se someterán a isquemia bilateral de 45 min y reperfusión posterior (I/R)

4) Ratas a las que se les administrara espironolactona 72 h antes de inducir I/R (Sp+I/R)

5) Ratas a las que se les inducirá I/R y que recibirán espironolactona 3 horas después de la isquemia.

Los animales se mantendrán durante 180 o 270 días en un ciclo de luz-obscuridad 12:12 y con acceso libre al alimento y agua. Cada 30 días se colocarán en jaulas metabólicas para recolección de orina de 24 h.

1. **Mediciones fisiológicas:** Se registrara el peso corporal, consumo de alimento y agua a lo largo del estudio, todos los animales se colocaran en jaulas metabólicas para la recolección de orina de 24 horas al inicio del protocolo y cada 30 días, cuantificando el volumen urinario, también se tomará una muestra de sangre para medir creatinina, osmolaridad, hematocrito, niveles séricos de, aldosterona, angiotensina I y renina, también se determinará la proteinuria y se registrara la presión arterial de los animales. Se determinara la tasa de filtración glomerular, flujo sanguíneo renal, electrolitos, se llevaran a cabo estudios histopatológicos y moleculares, cuantificación de lipoperoxidación de tejido renal.
   Filtración glomerular: Las ratas se anestesiaran con pentobarbital sódico (30 mg/kg de peso corporal i.p.). La preparación quirúrgica se realizará por medio de microcirugía de la siguiente forma: se colocará a la rata en la mesa termoregulada a 37 °C, se cateterizaran la tráquea con un tubo de polietileno PE-240, las venas yugulares, la arteria femoral con tubo de polietileno PE-50, y la vejiga con tubo de polietileno PE-90. Durante todo el experimento se registrará la presión arterial media en un polígrafo Grass. Las ratas se mantendrán en condiciones de euvolemia por la infusión de 10 ml/kg de plasma durante la cirugía seguida de una infusión de de azúcar baja en calorías (BC) al 5%[27] después de un periodo de equilibrio de 45 min, se recolectará la orina durante 30 minutos, tomándose muestras de sangre de la arteria femoral al inicio y al final de la

recolección. El volumen de sangre será reemplazado con sangre de una rata donadora por la vena yugular. La concentración de azúcar BC en plasma y orina se determinará por el método de Davidson [28].

Flujo Sanguíneo Renal (FSR): Una vez realizada la cirugía y que el animal se encuentre en condiciones de euvolemia, se disecará la arteria renal izquierda y se colocará una sonda conectada al flujometro Transonic System en donde se registrará en forma continua el FSR.

Sodio y Potasio: El sodio y el potasio se determinarán en suero y orina por el método ión-selectivo utilizando el aparato analizador de electrolitos Nova-4.

Estudios Histopatológicos: El riñón izquierdo será perfundido al finalizar el estudio a través de la arteria femoral con una solución de fosfatos y fijado con una solución de formol al 10 %, posteriormente será embebido en parafina y se realizaran cortes histológicos, se realizara una tinción de PAS y rojo de sirio, para determinar la presencia de arteriolopatía y el grado de fibrosis túbulo intersticial mediante microscopia de luz respectivamente. Además se cuantificara el grado de apoptosis mediante la técnica de túnel. Para estudiar si la proliferación celular se encuentra afectada se realizarán inmunotinciones para PCNA en los cortes de tejido renal.

Estudios Moleculares: Se extraerá el riñón derecho y se separa microscópicamente en corteza y médula renal y se congelará en nitrógeno líquido y se almacenará a -80°C hasta su procesamiento.

Extracción de RNA total: Se extraerá el RNA total de la corteza y medula renal por homogenización con tripiure. Una vez aislado el RNA, se determinará pureza y concentración mediante espectrofotometría y mediante un gel de agarosa al 1% se verificará la integridad del mismo. ***RT-PCR en tiempo real:*** Se llevará a cabo la transcripción reversa (TR)[29,30, 31] a partir del RNA total y posteriormente, la amplificación del DNAc mediante PCR en tiempo real para los genes TGF-beta, alfa-SMA, Hsp72, HIF1-alfa, Twist, Cinasa Rho, Kim-1, procaspasa 3, Bcl 2, Brcal, enzimas antioxidantes (glutatión peroxidasa, catalasa y superoxido dismutasa) y 18S, este ultimo como gen control mediante RT-PCR en tiempo real, utilizando oligonucleótidos específicos para cada gen y una sonda marcada con un fluoróforo para detectar la amplificación por PCR en tiempo real. Se calcularan los niveles relativos de expresión para cada uno de los genes antes mencionados en relación al gen control.

Análisis de Western Blot: Las proteínas serán extraídas por homogenización corteza y medula renal en 4 volúmenes de buffer de lisis (225mM manitol, 75mM de sacarosa, 0.1mM de EDTA, 0.5 mM de MOPS, 5 mM de benzamidina y 5mM de DTT), los homogenizados serán centrifugados a 4000 g por 8 min, se retirarán los restos celulares y se determinará la concentración de proteínas por el método de Lowry. Se cargaran 50mg de proteínas en un gel SDS- PAGE al 7.5%. Posteriormente, serán transferidas a una membrana PVDF, y se incubarán con anticuerpos específicos para E-caderina, alfa-SMA, Fibronectina, Colágena I y IV, TGF-β, Smad 2 y 3, así como el estado de fosforilación de Smad, la actina se utilizará como proteína control. Para estudiar la posible participación de las MAP cinasas y de la vía del NfkappaB, se determinaran los niveles de proteína de ERK 1 y de su estado de fosforilación así como del factor de transcripción NFkappaB y su forma fosforilada (activa). La cantidad de proteína se detectará mediante un ensayo de quimiolumnicencia por autorradiografía de las bandas, y se cuantificarán por análisis densitométrico.

Evaluación de los niveles de lipoperoxidación: En muestras de homogenado de tejido renal se determinaran los niveles de malondialdehido como marcador de la lipoperoxidación de membranas celulares, a través de la técnica de TBARS.

2. **Alimentación:** NO
3. **Agua:** NO
4. **Maniobras conductuales:** NO
5. **Modificaciones Ambientales:** Se emplearan cajas de policarbonato de piso sólido con camas de aserrín para cada una de las ratas, para su estancia en el bioterio, además será necesario el uso de jaulas metabólicas al inicio del tratamiento y 24 horas antes del sacrificio, para recolección de orina de 24 horas.
6. **Restricción física y ejercicio:** NO
7. **Inmunización:** NO

8. **Administración de medicamentos:** Se empleará anestésicos: como éter para recolectar muestras de sangre al inicio del tratamiento, ketamina: xilasina (dosis: 50/10) al momento de realizar los experimentos de determinación de tasa de filtración glomerular.
9. **Inoculación de agentes biológicos**: NO
10. **Uso de sustancias peligrosas**: NO
11. **Radiaciones**: NO
12. **Trauma**: NO
13. **Cirugía**: 10 ratas de cada grupo serán anestesiadas con ketamina/Xilasina (50/10 mg/Kg de peso corporal i.p.). La preparación quirúrgica se realizará por medio de microcirugía de la siguiente forma: se colocará a la rata en la mesa termoregulada a 37 °C, se cateterizaran la tráquea con un tubo de polietileno PE-240, las venas yugulares, la arteria femoral con tubo de
14. polietileno PE-50, y la vejiga con tubo de polietileno PE-90. Durante todo el experimento se registrará la presión arterial media en un polígrafo Grass. Las ratas se mantendrán en condiciones de euvolemia por la infusión de 10 ml/kg de plasma durante la cirugía seguida de una infusión de azúcar BC 5% y después de un periodo de equilibrio de 45 min, se recolectará la orina durante 30 minutos, tomándose muestras de sangre de la arteria femoral al inicio y al final de la recolección. El volumen de sangre será reemplazado con sangre de una rata donadora por la vena yugular.

    Otras106 ratas de cada grupo serán anestesiadas nuevamente con ketamina/Xilasina (50/10 mg/Kg de peso corporal i.p.). Se colocarán en una mesa termoregulada a 37 °C, se realizara una incisión media en el abdomen, posteriormente se removerá el riñón derecho, separándolo macroscópicamente en corteza y médula renal, se congelará en nitrógeno líquido y se almacenará a -80°C hasta su procesamiento para los estudios moleculares. Finalmente las ratas restantes de cada grupo se anestesiaran con el mismo analgésico y relajante muscular antes mencionado (50/10 mg/Kg de peso corporal i.p.). La preparación quirúrgica se realizará por medio de microcirugía de la siguiente forma: se colocará a la rata en la mesa termoregulada a 37 °C, se cateterizaran la tráquea con un tubo de polietileno PE-240 y una arteria femoral con tubo de polietileno PE-50, posteriormente serán perfundidos los riñones a través de la arteria femoral con una solución de fosfatos y fijados con una solución de formol al 10 %, posteriormente serán embebidos en parafina y se realizaran cortes histológicos, para determinar los cambios histológicos característicos del modelo experimental.

    Obtención de muestras: Se recolectara orina de 24 horas mediante la colocación de los animales en jaulas metabólicas, Volumen: 4ml, Conservación: -20°C, Frecuencia: Basal y cada 30 días, condiciones previas: ninguna, Se recolectara muestra de sangre al inicio del tratamiento a través de seno ocular usando capilar, al finalizar el estudio a través de la arteria femoral, Volumen: 1ml, Conservación: -20°C, Frecuencia: Basal y al finalizar los 36 días, condiciones previas: ninguna.

15. **Obtención de tejidos**: Se obtendrán los riñones a través de escisión quirúrgica, bajo anestesia.
16. **Eutanasia**: se llevara a cabo a través de una sobredosis de anestesia por vía intravenosa.
17. **Disposición de cadáveres**: será determinado de acuerdo con lo establecido en la NOM-087-ECOL-1995

Análisis estadístico

Los resultados obtenidos se analizarán por ANOVA de una vía y las diferencias entre grupos se determinarán con la prueba de Student-Neumann-Kuels. Los datos se presentarán como el promedio ± el error estándar.

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