Original Article

Angiotensin II Increases Intrarenal Transforming Growth Factor-β1 in Rats Submitted to Sodium Overload Independently of Blood Pressure

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Angiotensin II (Ang II) promotes sodium-retention, cell growth and fibrosis in addition to its classical effects on blood pressure and fluid homeostasis. In this study we examined whether low and non-hypertensive doses of exogenous Ang II could enhance the intrarenal expression of transforming growth factor-β1 (TGF-β1) observed in rats submitted to sodium overload. Sprague-Dawley-rats were infused for 2 h with 0.1 and 5 μg kg^{-1} h^{-1} Ang II (Ang 0.1 and Ang 5, respectively) together with saline solution at four different concentrations (isotonic and Na 0.5 mol L^{-1}, Na 1.0 mol L^{-1} and Na 1.5 mol L^{-1}). Renal function and mean arterial blood pressure (BP) were measured. The renal distributions of TGF-β1, α-smooth-muscle-actin (α-SMA) and nuclear factor-κB (NF-κB) were evaluated by immunohistochemistry. While the Ang 0.1 groups were normotensive, the Ang 5 groups developed arterial hypertension progressively, and the highest blood pressure values were observed when rats were simultaneously infused with Na 1.5 mol L^{-1}. Glomerular function was not altered in any group. In cortical tubules, all groups infused with Ang II (0.1 and 5) and hypertonic saline solution (HSS) showed an increase in TGF-β1 immunostaining compared to those infused with HSS alone. In medullary tubules, only the Ang 5–Na 0.5 group showed a significant increase in TGF-β1 immunostaining compared to the Na 0.5 group. Peritubular positive staining for α-SMA was present in groups receiving Ang alone or Ang-Na, in a sodium concentration–dependent manner. In cortical-tubules, NF-κB immunostaining was significantly increased in the Ang groups in comparison with the control and in Ang–Na 0.5 and Ang–Na 1.0 groups in comparison with the Na 0.5 mol L^{-1} and Na 1.5 mol L^{-1} groups, respectively, except in the case of the Ang 0.1–Na 1.5 mol L^{-1} and Ang 5–Na 1.5 mol L^{-1} groups. Moreover, Ang II and sodium overload induced additional changes in TGF-β1, α-SMA and NF-κB immunostanding in glomeruli, medullary tubules and renal vessels. In conclusion, the interaction of Ang II with acute-sodium overload exacerbated intrarenal TGF-β1, α-SMA and NF-κB expression, independently from changes in blood pressure levels, in normal rats. (*Hypertens Res* 2008; 31: 707–715)

***Key Words*: sodium, angiotensin II, transforming growth factor-β1, kidney**

Introduction

Transforming growth factor-β1 (TGF-β1), belongs to a family of five polypeptides that exert complex effects on organ development, cell growth and differentiation, expression of extracellular matrix, angiogenesis and tissue repair. It has been implicated as an important factor for the development of

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Table 1. Parameters Evaluated in Urine and Blood at the End of the Study

	PL Na (mEq L^{-1})	U_V (μL min^{-1})	$U_{Na}V$ (μmol min^{-1})	U_KV (μmol min^{-1})
C	138±2	6.2±0.4	0.35±0.09	0.93±0.17
Na 0.5	147±1	36.8±3.5	10.85±1.67	4.07±0.28
Na 1.0	151±1	105.1±15.4	32.87±3.69	4.57±0.55
Na 1.5	161±2	147.0±13.2	42.59±3.21	6.16±0.47
Ang 0.1	144±1	2.2±0.6[a]	0.01±0.01[a]	0.63±0.24
Ang 0.1–Na 0.5	145±1	28.9±5.1	6.39±0.94	4.04±0.43
Ang 0.1–Na 1.0	150±2	101.9±6.3	29.93±3.83	4.47±0.44
Ang 0.1–Na 1.5	158±2	106.5±25.5	34.79±4.94	5.35±1.06
Ang 5	140±2	7.5±1.8	0.44±0.15	1.15±0.36
Ang 5–Na 0.5	146±3	18.2±3.0[b]	4.92±1.33[b]	2.62±0.35[b]
Ang 5–Na 1.0	152±2	47.3±11.4[c]	14.10±3.10[c]	3.86±0.42
Ang 5–Na 1.5	156±4	116.3±9.7[d]	35.92±2.62	5.47±0.50

PL Na, plasmatic sodium; U_V, urine flow rate; $U_{Na}V$, sodium urinary excretion; U_KV, potassium urinary excretion; C, control; Na 0.5 (1.0, 1.5), NaCl 0.5 (1.0, 1.5) mol L^{-1}; Ang 0.1 (5), angiotensin II 0.1 (5) μg kg^{-1} h^{-1}. Values are mean±SEM. [a]$p<0.05$ *vs*. C; [b]$p<0.05$ *vs*. Na 0.5; [c]$p<0.05$ *vs*. Na 1.0; [d]$p<0.05$ *vs*. Na 1.5.

renal fibrosis, glomerulosclerosis and tubular atrophy associated with salt loading, and it has been shown to be overexpressed in salt-loaded rats (*1*). Renal salt overload inhibits renin release from the juxtaglomerular apparatus and reduces circulating angiotensin II (Ang II). However, experimental studies have demonstrated that, despite the inhibition of the renin-angiotensin system (RAS) by salt-overload, local Ang II may be stimulated (*2*). Ang II, in addition to playing a critical role on blood pressure (BP) and hydrosaline balance regulation in the kidney, exerts direct effects at the cellular level, promoting cell growth and differentiation, and is a mediator of oxidative stress, inflammation and fibrosis (*3*, *4*). Nuclear factor-κB (NF-κB) and AP-1 are the major nuclear transcription factors responsible for the regulation of several inflammatory genes in response to Ang II, such as those for cytokines, chemokines, and adhesion molecules (*2*). *In vitro*, increased activation of NF-κB in response to Ang II has been shown in several cell types, including vascular smooth muscle cells and endothelial, glomerular, tubular and mononuclear cells (*2*). *In vivo*, increased NF-κB activity and expression are shown in the vasculature, heart, and kidney of Ang II–infused rats (*5*). In addition, there exists a close interaction between the RAS and TGF-β1 systems. Recent data provide compelling evidence that other components of the RAS, including Ang III, renin and aldosterone, also activate the TGF-β1 system (*6*). Ang II–stimulated cells express more TGF-β1 mRNA and produce active TGF-β1. Ang II also enhances post-transcriptional expression of TGF-β1 protein (*7*, *8*). More recently, it has been shown that TGF-β1 stimulates angiotensinogen gene expression, and this stimulation is mediated by the generation of reactive oxygen species (ROS), suggesting that Ang II and TGF-β1 may form a positive feedback loop to enhance their respective gene expressions, leading to renal injury (*9*). α-Smooth muscle actin (α-SMA) is a smooth muscle cell cytoskeleton protein and a marker of trans-differentiation from fibroblast to myofibroblast. In the normal kidney, α-SMA is present only in the vascular wall, whereas during the fibrotic process, cells expressing α-SMA are detected in the renal interstitium. α-SMA is strongly upregulated by TGF-β1, which plays a role in the transformation of fibroblasts into myofibroblasts in the tubulointerstitial area. In this regard, previous studies from our laboratory showed that an acute salt loading in normal rats increased intrarenal Ang II, TGF-β1 and α-SMA immunoexpression, even before structural and functional changes appeared, and these upregulations were correlated with greater sodium tubular reabsorption (*10*). Considering that Ang II is able to enhance intrarenal TGF-β1 by a direct action or indirectly through an increase in sodium transport, it is possible to speculate that co-infusion of low-doses of exogenous Ang II together with hypertonic saline solution could exacerbate immunoexpression of the profibrogenic cytokine TGF-β1.

Methods

Male Sprague-Dawley rats (10–12 weeks old; 270–350 g body weight) were used. Animals were housed at a controlled temperature (23±2°C) and exposed to a daily 12-h light–dark cycle (lights on 7:00 AM to 7:00 PM) with free access to tap water and standard rat chow.

On the day of the experiment, rats were anesthetized with 10% urethane (1.2 g kg^{-1} body weight [b.w.] intraperitoneally), a tracheotomy was performed, and a PE-90 tube (3 cm long) was inserted into the trachea to maintain an open airway. The left femoral vein was catheterized with a Silastic cannula (0.12 mm i.d.) for continuous infusion. The right carotid artery was also catheterized with a T4 tube for blood sampling and continuous mean arterial pressure measurement, by means of a Statham GOULD P23ID transducer coupled to a Grass Polygraph 79D. The bladder was cannulated

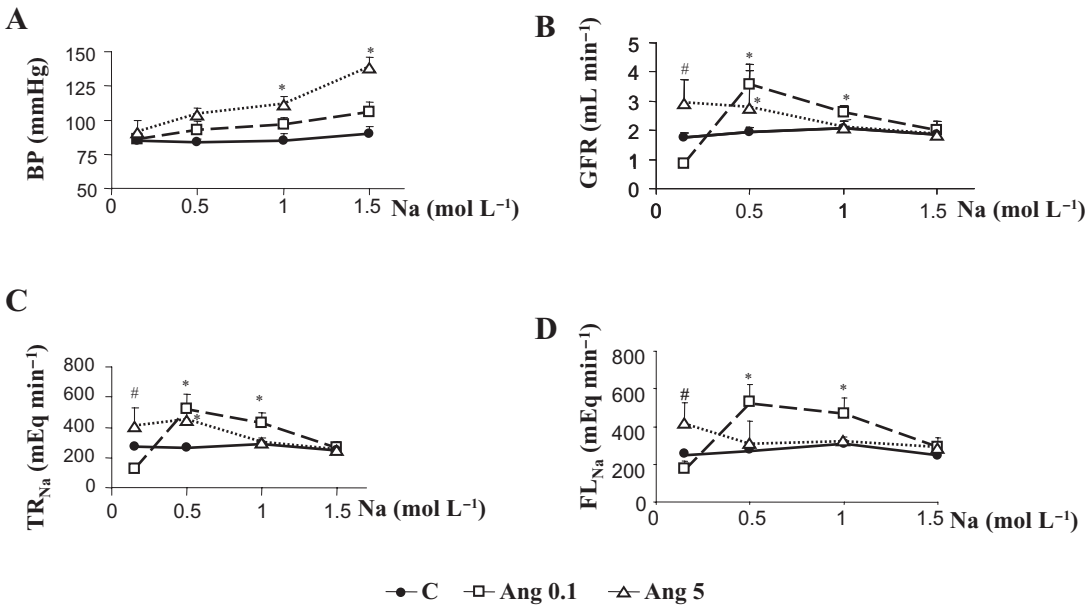

Fig. 1. *A: Blood pressure (BP). B: Glomerular filtration rate (GFR). C: Sodium tubular reabsorption (TR_{Na}). D: Sodium filtered load (FL_{Na}). C (control), Na^+ 0.15 mol L^{-1} (ISS); Ang 0.1, Ang II 0.1 μg kg^{-1} h^{-1}; Ang 5, Ang II 5 μg kg^{-1} h^{-1}. *$p < 0.05$ vs. Na groups; #$p < 0.05$ vs. Ang 0.1. Values are expressed as the means ± SEM. $n = 5–8$.*

for urine collection using a PE-75 cannula.

All infusions were carried out at the same rate of 0.04 mL min^{-1} using a syringe infusion pump (Sage Instruments, Orion Research, Boston, USA). A 45-min infusion with isotonic saline solution (ISS) was sufficient for steady state diuresis and urine collection. Then, to induce an acute sodium overload, one of four different solutions (an ISS [NaCl 0.15 mol L^{-1}]) or one of three hypertonic saline solutions (HSS) (NaCl 0.5 mol L^{-1}, 1.0 mol L^{-1} or 1.5 mol L^{-1}) and two non-hypertensive doses of Ang II (0.1 and 5 μg kg^{-1} h^{-1}) were infused over a period of 120 min. The following groups were thus established: 1) a control group infused with ISS (C); and experimental groups infused with 2) NaCl 0.5 mol L^{-1} (Na 0.5); 3) NaCl 1.0 mol L^{-1} (Na 1.0); 4) NaCl 1.5 mol L^{-1} (Na 1.5); 5) Ang II 0.1 μg kg^{-1} h^{-1} (Ang 0.1); 6) Ang II 5 μg kg^{-1} h^{-1} (Ang 5); 7) Ang 0.1–Na 0.5; 8) Ang 0.1–Na 1.0; 9) Ang 0.1–Na 1.5; 10) Ang 5–Na 0.5; 11) Ang 5–Na 1.0 and 12) Ang 5–Na 1.5.

Urine and blood samples were collected at 30 and 60 min, respectively, for sodium, potassium and creatinine measurements. Mean arterial BP was monitored continuously throughout the procedure.

Urine and Blood Measurements

Urine and plasma sodium, potassium and creatinine were measured by standard methods. Sodium fractional excretion and creatinine clearance were calculated according to the standard formulas. Creatinine clearance was assessed in order to evaluate the glomerular filtration rate (GFR). Urinary flow was expressed as μL min^{-1}; plasma and urinary sodium and potassium as μEq L^{-1}; sodium and potassium urinary excretion as μmol min^{-1}; and GFR as mL min^{-1}.

Kidney Processing for Histological Examination

At the end of the infusion period, the left kidney was perfused with ISS through the abdominal aorta until the blood was washed out and the parenchyma had a pale appearance. The kidney was then rapidly excised, decapsulated, longitudinally cut and harvested for light microscopy and immunohistochemical studies.

Tissues were fixed in phosphate-buffered 10% formaldehyde (pH 7.2) and embedded in paraffin using conventional histological techniques. Three μm-thick sections were cut and stained with hematoxylin-eosin for light microscopy. For immunohistochemistry, sections were deparaffined and rehydrated, and endogenous peroxidase activity was blocked by treatment with 0.5% H_2O_2 in methanol for 20 min. Local TGF-β1 was detected using the following antibodies: mouse anti-TGF-β1 (Santa Cruz Biotechnology Inc., Santa Cruz, USA; dilution: 1:200), mouse anti-α SMA (Sigma Chemical Co., St. Louis, USA; dilution: 1:150) and mouse anti–NF-κB p65 (Santa Cruz Biotechnology Inc.; dilution: 1:150). Immunostaining was carried out by means of a commercial modified avidin-biotin-peroxidase complex technique (Vectastain ABC Universal Elite Kit; Vector Laboratories, Burlingame, USA) and counterstained with hematoxylin. The samples were handled as previously described (*11*).

Histological sections were observed using a Nikon E400

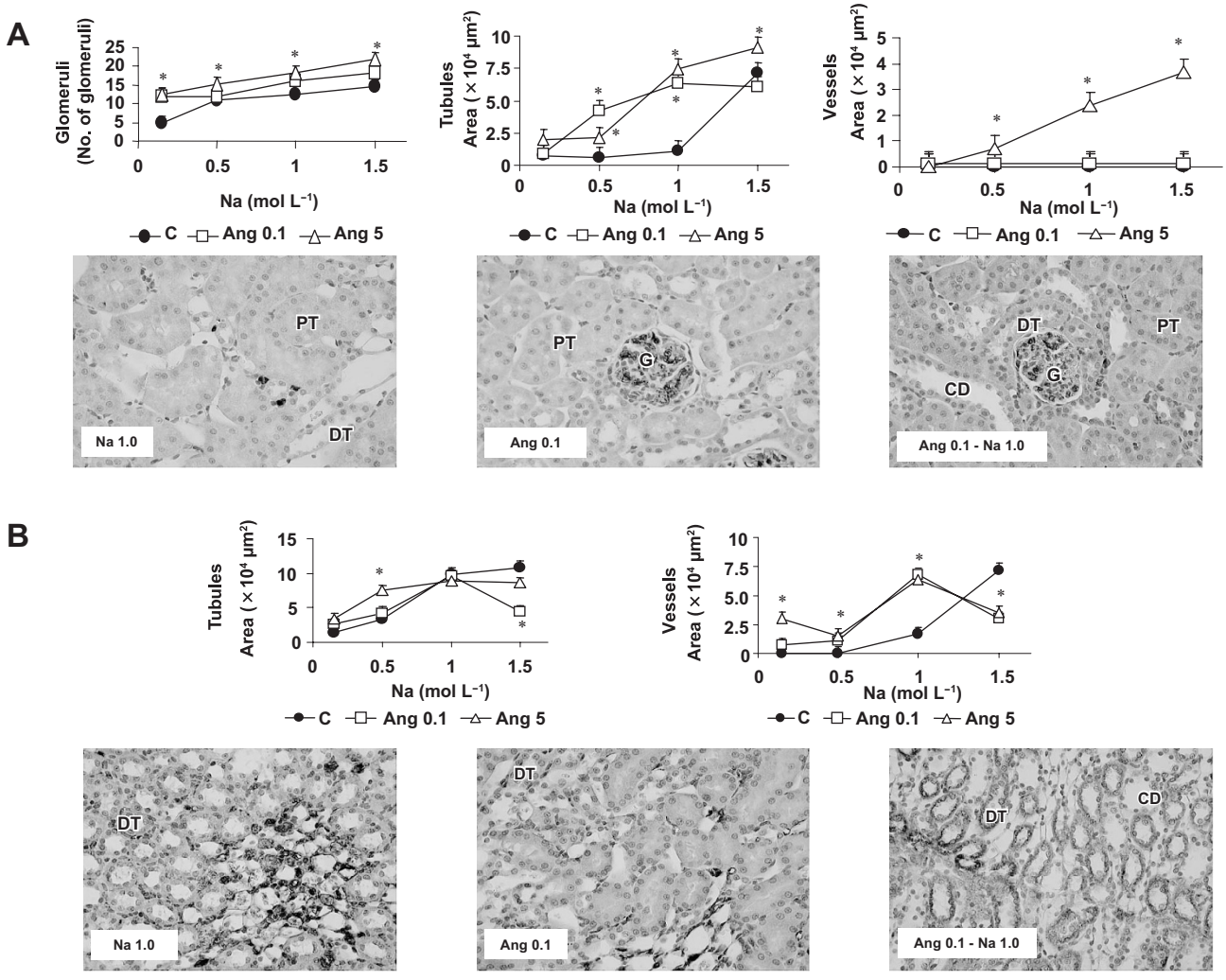

Fig. 2. *TGF-β1. Immunostaining in the renal cortex (A) and medulla (B). A: Quantitative representation of positive staining in glomeruli (expressed as the number of positive glomeruli on a counted total of 50 glomeruli in each slice±SD) and in vessels and tubules (expressed as area: ×10⁴ μm²±SD). C (control), Na⁺ 0.15 mol L⁻¹ (ISS); Ang 0.1, Ang II 0.1 μg kg⁻¹ h⁻¹; Ang 5, Ang II 5 μg kg⁻¹ h⁻¹. *p < 0.01 vs. the respective control. n = 5. Representative photomicrograph of TGF-β1 in the renal cortex (original magnification ×400): Na 1.0, Na⁺ 1.0 mol L⁻¹; Ang 0.1, Ang II 0.1 μg kg⁻¹ h⁻¹; Ang 0.1–Na 1.0, Ang II 0.1 μg kg⁻¹ h⁻¹–Na⁺ 1.0 mol L⁻¹. G, the glomerulus; PT, proximal tubules; DT, distal tubules. B: Quantitative representation of positive staining in vessels and tubules (expressed as area: ×10⁴ μm²±SD). C (control), Na⁺ 0.15 mol L⁻¹ (ISS); Ang 0.1, Ang II 0.1 μg kg⁻¹ h⁻¹; Ang 5, Ang II 5 μg kg⁻¹ h⁻¹. *p < 0.01 vs. the respective control. n = 5. Representative photomicrograph of TGF-β1 in renal medulla (original magnification ×400): Na 1.0, Na⁺ 1.0 mol L⁻¹; Ang 0.1, Ang II 0.1 μg kg⁻¹ h⁻¹; Ang 0.1–Na 1.0, Ang II 0.1 μg kg⁻¹ h⁻¹+Na⁺ 1.0 mol L⁻¹. DT, distal tubules; CD, collecting ducts.*

light microscope (Nikon Instrument Group, Melville, USA). Ten consecutive microscopic fields per sample were analyzed at 100× magnification to evaluate morphological changes in glomeruli and tubules, as well as in the renal interstitium. All measurements were carried out using image analysis software (Image-Pro Plus ver. 4.5 for Windows; Media Cybernetics, LP, Silver Spring, USA). Immunostaining for TGF-β1 is expressed as the positively stained area/1,408,593 μm²±SD.

Statistical Analysis

Results from urine and blood measurements and mean arterial pressure levels are expressed as the means±SEM. The Gaussian distribution was evaluated by the Kolmogorov and Smirnov method, and comparisons among groups were carried out using ANOVA followed by the Newman-Keuls test. Statistical analysis for those parameters with non-Gaussian distribution (such as histological data) was performed by the

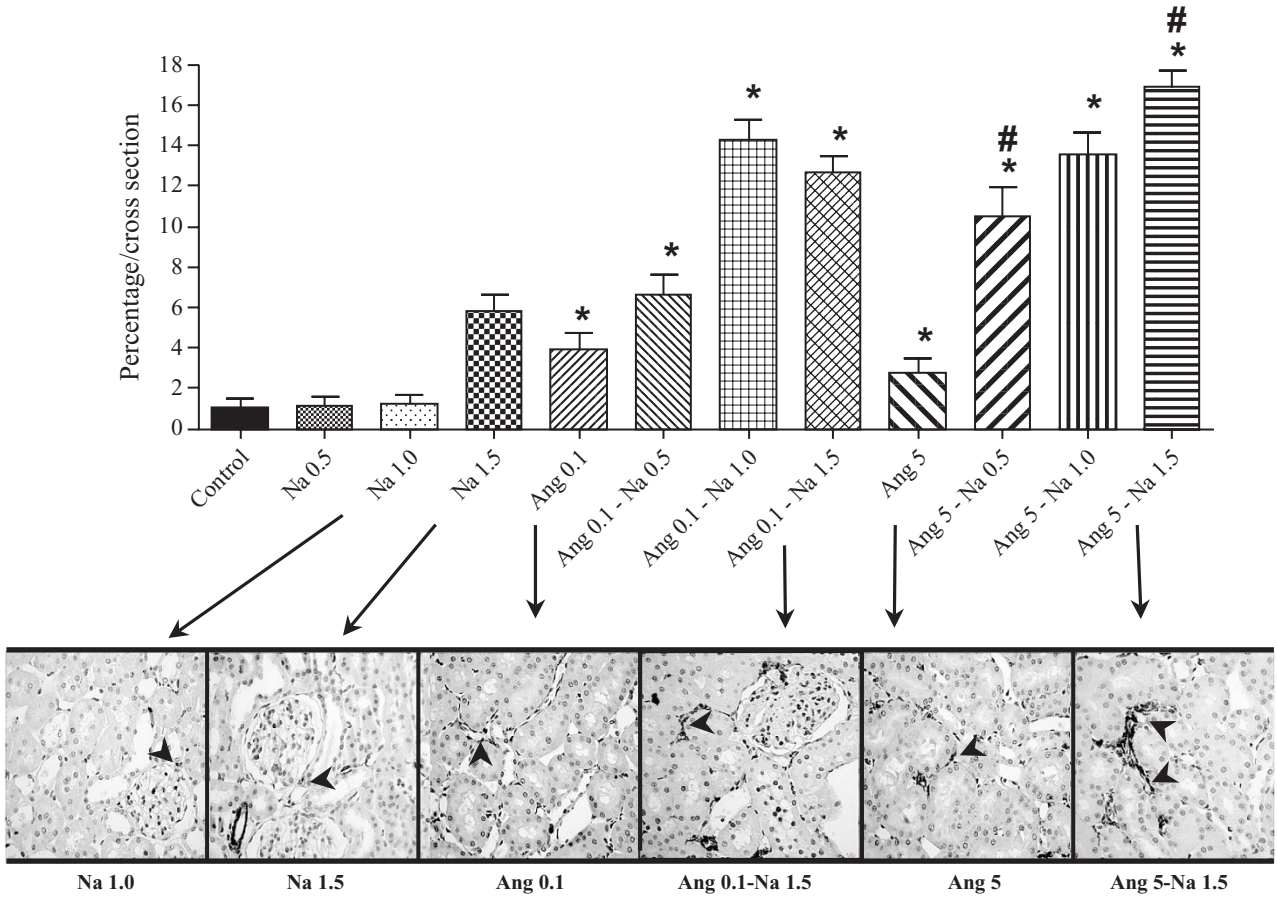

Fig. 3. *α-Smooth muscle actin (α-SMA). Immunostaining in the renal cortex. The upper panel indicates quantitative representation of positive staining in the renal interstitium and tubular epithelial cells (expressed as area: $\times 10^4$ $\mu m^2 \pm SD$). *$p < 0.01$ vs. Na groups; #$p < 0.01$ vs. Ang 0.1–Na groups. The lower panel illustrates the positive area of α-SMA (arrows) in each group (original magnification ×400).*

Kruskal-Wallis test (Nonparametric ANOVA) and Dunn's multiple comparison test; and processed through GraphPad Prism, version 2.0 (GraphPad Software, Inc., San Diego, USA). Values of $p < 0.05$ were considered statistically significant. All the results mentioned throughout the study were statistically significant. Results of groups infused with Ang II alone were compared with controls infused with ISS. Results of groups infused with Ang II plus HSS were compared with those groups infused with HSS at the same molarity (*e.g.*, Ang 0.1–Na 0.5 or Ang 5–Na 0.5 *vs*. Na 0.5; Ang 0.1–Na 1.0 or Ang 5–Na 1.0 *vs*. Na 0.1; and Ang 0.1–Na 1.5 or Ang 5–Na 1.5.*vs*. Na 1.5).

Results

To assess the effects of sodium overload and exogenous Ang II infusion on BP and glomerular function, BP was measured throughout the experimental period, and urinary flow, plasma and urinary sodium and potassium, and creatinine levels were determined.

Table 1 summarizes the plasma sodium concentration, urinary sodium and potassium excretion, and urinary flow at the end of the study. The plasma sodium concentration increased in a sodium concentration–dependent manner, independently of the presence or absence of exogenous Ang II. Urinary flow increased in a sodium concentration–dependent manner. The Ang 5 plus salt overload group showed a decrease in diuresis as compared with the salt overload groups without Ang II. Urinary flow and urinary sodium excretion showed a similar behavior. Urinary potassium excretion was not significantly different among the groups, with the exception of a significant decrease in the Ang 5–Na 0.5 group.

Figure 1 shows the results for BP, GFR, sodium tubular reabsorption (TR_{Na}) and sodium filtered load (FL_{Na}). The results shown are the values measured at the end of the study. BP increased only in groups Ang 5–Na 1.0 and Ang 5–Na 1.5 compared to Na 1.0 and Na 1.5, respectively (Fig. 1A). Infusion of Ang 0.1 or Ang 5 alone did not elevate BP compared with the control group infused with ISS.

GFR increased in the Ang 5 group compared to the Ang 0.1

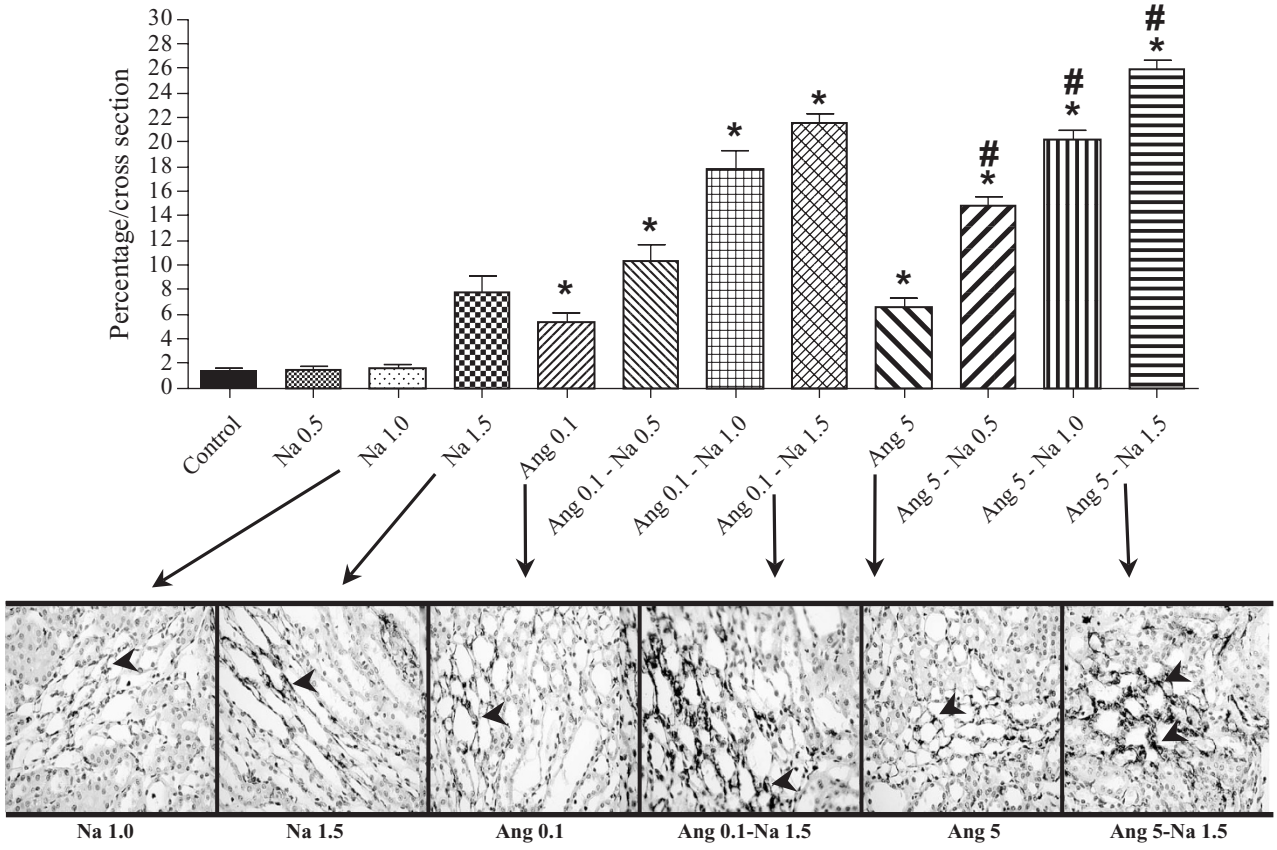

Fig. 4. *α-Smooth muscle actin (SMA). Immunostaining in the renal medulla. The upper panel indicates the quantitative representation of positive staining in the renal interstitium and tubular epithelial cells (expressed as area: $\times 10^4$ $\mu m^2 \pm SD$). $^*p < 0.01$ vs. Na groups; $^{\#}p < 0.01$ vs. Ang 0.1–Na groups. The lower panel illustrates the positive area of SMA (arrows) in each group (original magnification ×400).*

group, and in the Ang 5–Na 0.5, Ang 1–Na 0.5 and Ang 0.1–Na 1.0 groups compared to those infused with HSS alone (Fig. 1B).

TR_{Na} (Fig. 1C) and FL_{Na} (Fig. 1D) exhibited a very similar response pattern to that of GFR, being significantly higher in the Ang 5 group compared to the Ang 0.1 group, and in the Ang 0.1–Na 0.5 and Ang 0.1–Na 1.0 groups compared with Na 0.5 and Na 1.0, respectively. Additionally, the Ang 5–Na 0.5 group showed a greater TR_{Na} than the Na 0.5 group. Histological examination did not reveal morphological damage in the glomeruli and tubules in any group (data not shown).

Figure 2 shows the TGF-β1 immunostaining in the renal cortex (Fig. 2A) and the medulla (Fig. 2B). Panel A shows staining in the renal cortex of rats infused with Ang II (0.1 and 5 $\mu g\ kg^{-1}\ h^{-1}$) with or without HSS. In glomeruli, TGF-β1 was markedly elevated in the group receiving Ang 5 alone compared to the C group and in the 3 groups co-infused with Ang 5 and sodium compared with the 3 HSS groups. In cortical tubules, all groups with Ang II (0.1 and 5 $\mu g\ kg^{-1}\ h^{-1}$) and HSS exacerbated TGF-β1 immunostaining in a sodium concentration–dependent manner compared to those with HSS alone, except in the case of the Ang 0.1–Na 1.5 group *vs*. the Na 1.5 group. In medullary tubules, only the group Ang 5–Na 0.5 showed increased TGF-β1 immunostaining compared to the group Na 0.5. In vessels, only the highest Ang II dose induced stimulation of TGF-β1 expression, and this stimulation was also sodium-dependent.

In medullary tubules (Fig. 2B), TGF-β1 immunostaining was increased in the Ang 5–Na 0.5 group compared to the Na 0.5 group. Furthermore, the Ang 5–Na 1.5 group showed a decrease in TGF-β1 immunostaining compared to the Na 1.5 group. In medullary vessels, TGF-β1 was markedly elevated in the Ang 5, Ang 5–Na 0.5, Ang 5–Na 1.0, and Ang 0.1–Na 1.0 groups compared to both the C and HSS groups. In addition, the Ang 0.1–Na 1.5 and Ang 5–Na 1.5 groups showed a decreased response, compared to the Na 1.5 group. TGF-β1 staining was present in the glomerular mesangium, glomerular capillary endothelium and vascular endothelium. TGF-β1 staining was very scarce in podocytes, especially in the Na 0.5 group. Tubular cytoplasmic staining was observed in the collecting and distal tubules. Positive expression in proximal tubules was present only in the Ang 5 and Ang 0.1 groups

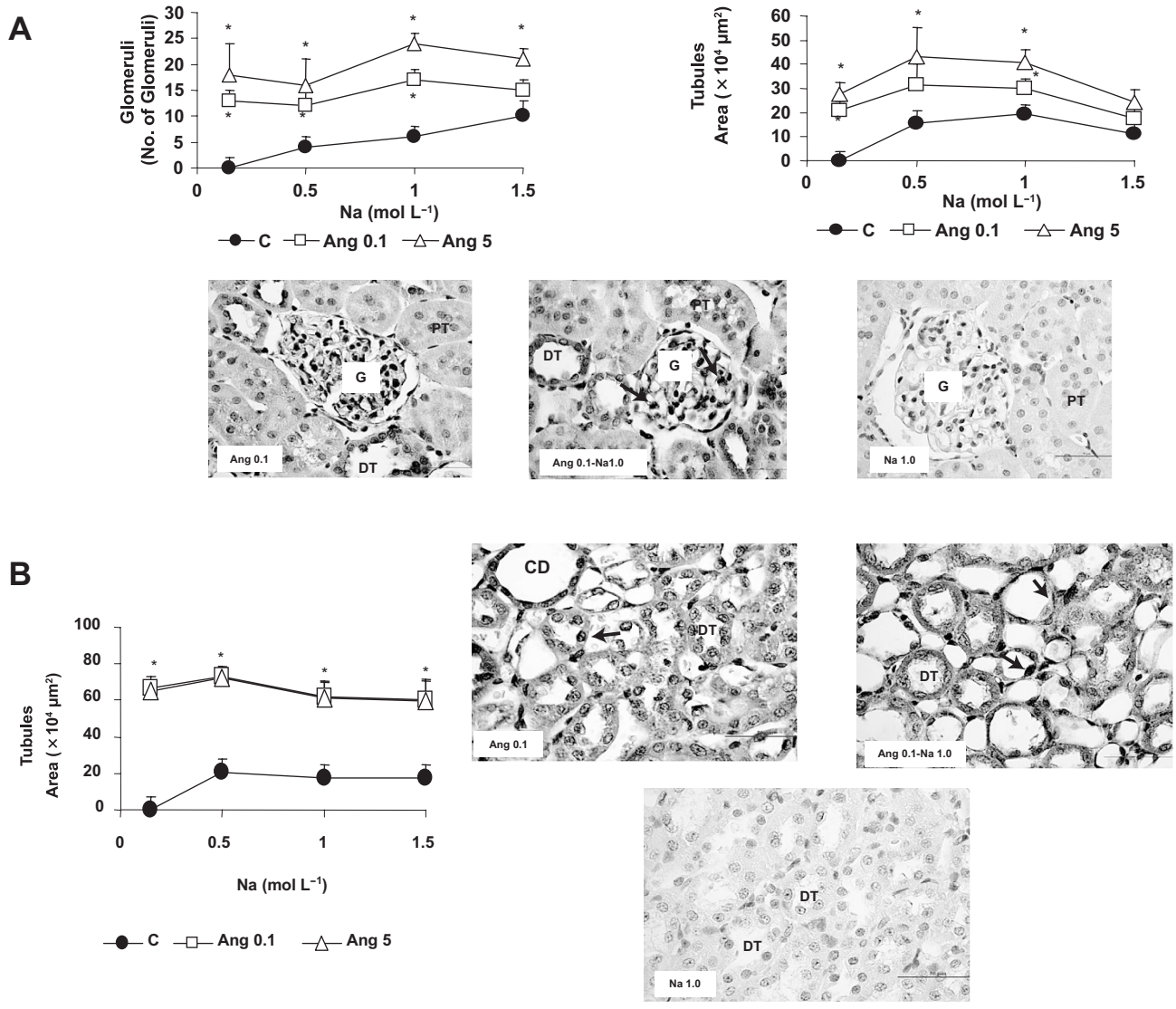

Fig. 5. *NF-κB. Immunostaining in the renal cortex (A) and medulla (B). A: Quantitative representation of positive staining in the glomeruli, expressed as the number of positive glomeruli on a counted total of 50 glomeruli in each slice±SD). C (control), Na^+ 0.15 mol L^{-1} (ISS); Ang 0.1, Ang II 0.1 μg kg^{-1} h^{-1}; Ang 5, Ang II 5 μg kg^{-1} h^{-1}. *$p < 0.01$ vs. the respective control. $n = 5$. Quantitative representation of positive staining tubules, expressed as area (×10^4 μm^2±SD). C (control), Na^+ 0.15 mol L^{-1} (ISS); Ang 0.1, Ang II 0.1 μg kg^{-1} h^{-1}; Ang 5, Ang II 5 μg kg^{-1} h^{-1}. *$p < 0.01$ vs. the respective control. $n = 5$. Representative photomicrograph of NF-κB in the renal cortex (original magnification ×600): Na 1.0, Na^+ 1.0 mol L^{-1}; Ang 0.1, Ang II 0.1 μg kg^{-1} h^{-1}; Ang 0.1–Na 1.0, Ang II 0.1 μg kg^{-1} h^{-1}+Na^+ 1.0 mol L^{-1}. G, the glomerulu; PT, proximal tubules; DT, distal tubules. Arrows indicate positive staining for p65 subunit NF-κB in the nucleus of epithelial tubular cells. B: Quantitative representation of positive staining in tubules, expressed as area (×10^4 μm^2±SD). C (control): Na^+ 0.15 mol L^{-1} (ISS); Ang 0.1, Ang II 0.1 μg kg^{-1} h^{-1}; Ang 5, Ang II 5 μg kg^{-1} h^{-1}. *$p < 0.001$ vs. the respective control. $n = 5$. Representative photomicrograph of NF-κB in the renal medulla (original magnification ×600): Na 1.0, Na^+ 1.0 mol L^{-1}; Ang 0.1, Ang II 0.1 μg kg^{-1} h^{-1}; Ang 0.1–Na 1.0, Ang II 0.1 μg kg^{-1} h^{-1} +Na^+ 1.0 mol L^{-1}. DT, distal tubules; CD, collecting ducts. Arrows indicate positive staining for p65 subunit NF-κB in the nucleus of epithelial tubular cells.*

with higher Na concentrations.

Figures 3 and 4 show the α-SMA immunostaining in the renal cortex and medulla, mainly in the peritubular area, of rats infused with Ang II (0.1 or 5 μg kg^{-1} h^{-1}) with or without HSS. α-SMA immunostaining in the cortex and medulla increased in the groups receiving Ang alone or in those receiving both Ang and Na in a sodium concentration–dependent manner.

Figure 5 shows NF-κB immunostaining in the renal cortex and medulla of rats infused with Ang II (0.1 and 5 μg kg^{-1} h^{-1}) with or without HSS. Positive immunostaining for NF-κB was seen in the nuclei and cytoplasm of tubular and glomerular cells. In the glomeruli, NF-κB staining was increased in the Ang group compared with the C group and in the Ang-Na groups compared with the HSS groups, with the exception of the Ang 0.1–Na 1.5 group. In the cortical tubules (Fig. 5A), NF-κB immunostaining increased in the Ang groups compared to the C group and in the Ang 0.1–Na 0.5 and Ang 0.1–Na 1.0 groups compared to Na 0.5 group and in the Ang 5–Na 1.0 and Ang 5–Na 1.0 compared to the Na 1.0 group, with the exception of the Ang 0.1–Na 1.5 and Ang 5–Na 1.5 groups. In the medullary tubules (Fig. 5B), NF-κB was higher in the Ang and Ang-Na groups than in either the C or HSS group.

Discussion

In this study, acute administration of low doses of Ang II further enhanced the intrarenal TGF-β1, α-SMA and NF-κB expression in rats submitted to sodium overload, independently from changes in BP. The low Ang II dose, administered alone or together with hypertonic HSS, did not raise BP, but increased TGF-β1 expression in the renal cortex as well as the renal medulla. However, the high Ang II dose administered alone or together with hypertonic Na 0.5 did not alter BP, but increased TGF-β1 expression as well. BP was only elevated by the Ang 5 dose when it was simultaneously co-infused with high sodium overload (Na 1.0 and 1.5), showing a progressive enhancement of TGF-β1 expression in the cortex but not in the renal medulla. Thus our results demonstrated the absence of a correlation between BP levels and TGF-β1 expression in groups with lower doses of Ang II, suggesting that TGF-β1 expression may be regulated by other factors.

In the present study, GFR was increased or unchanged, rather than decreased. Co-infusion of Ang II and ISS augmented GFR in the Ang 5 group compared to the Ang 0.1 group. It is known that the Ang II at a low concentration (10^{-12} mol L^{-1}) is able to increase sodium transport at the macula densa, leading to a decrease in GFR, and then a decrease in urine flow velocity (U_V) and sodium urinary excretion ($U_{Na}V$). Nevertheless, when Ang 5 was infused, GFR and TR_{Na} increased but U_V and $U_{Na}V$ were similar to those in the control group. Moreover, a lower Ang II dose, co-infused together with HSS (Na 0.5 mol L^{-1} or Na 1.0 mol L^{-1}), was able to increase GFR and sodium tubular reabsorption, whereas the higher Ang II dose co-infused with the same HSS did not change GFR, although it reduced the urinary sodium excretion. These data suggest that the early high GFR could be necessary to exert a natriuretic influence, in order to maintain normal BP and sodium balance by counteracting sodium-retaining factors such as Ang II. Although this may serve to maintain sodium balance in the short term, using this mechanism over the long-term or at higher Ang doses would clearly be maladaptive by contributing to progressive glomerular injury. Thus, the decline of GFR to progressively more normal levels is shown to be a renal function change for the purpose of retaining sodium and thereby increasing BP, as we observed in the Ang 5–Na 1.0 and Ang 5–Na 1.5 groups. Therefore, the increase in TGF-β1 expression in the cortical tubules induced by both doses of Ang II alone and by both doses of Ang II with Na 1.0 and Na 1.5 could be related to the fact that both GFR and sodium tubular transport were increased. The present data suggest that increased activation of TGF-β1 in the kidney could be the result of augmented GFR and/or sodium tubular reabsorption when low doses of Ang II are infused simultaneously with sodium overload. Accordingly, it has been demonstrated that an increment in tubular sodium concentration or sodium flow leads to a greater sodium transport. This effect has been associated with enhanced production of superoxide anion and decreased production of nitric oxide, and these changes are dependent on oxygen utilization (*12*). Since either a reduction in nitric oxide or an increase of superoxide anion could regulate TGF-β1 expression, nitric oxide provides an essential inhibitory feedback mechanism that modulates TGF-β1 production. Glomerular and vascular ring preparations incubated with L-NAME, a NOS3 inhibitor, have been shown to produce increased amounts of TGF-β1, while incubation with a nitric oxide donor decreases TGF-β1 production (*1*). Additionally, enhanced superoxide anion is known to act as a second messenger in the activation of transcription nuclear factors NF-κB and activator protein-1 (AP-1), which plays a key role in the generation of proinflammatory cytokines, chemokines and growth factors such as TGF-β1 (*13*). In addition to the sodium-retaining effect, Ang II activity has other potential consequences, including production of superoxide anion by NAD(P)H oxidase activation, which is mainly mediated *via* Ang II type 1 (AT1) receptors (*14*). Therefore, considering the extensive localization of AT1 receptors in the luminal and basolateral membranes of proximal and distal nephron segments (*5*), Ang II can regulate the activation of transcriptional factors through superoxide anion produced directly by NAD(P)H oxidase-activation or through superooxide anion produced indirectly through increased sodium transport (*15*).

In the present study, the higher NF-κB expressions observed in the glomeruli and tubules of the Ang-Na groups compared to the Na groups were independent of the BP level.

A comparison of the NF-κB and TGF-β1 expression profiles found in our study suggests that the greater TGF-β1 expression in the glomeruli and tubules in the Ang-Na groups may have been due to the activation of NF-κB, except in the case of the groups that developed arterial hypertension, in which some other mechanism of activation could be present.

In the present study, we observed a higher rate of positive staining for α-SMA in the renal peritubular interstitium of the Ang and Ang-Na groups than in that of the Na groups. The accumulation of myofibroblasts, which express α-SMA and thereby lead to extracellular matrix expansion, constitutes an early event in interstitial fibrogenesis. Since transformation of

fibroblasts into myofibroblasts in the tubulointerstitial area is strongly up-regulated by TGF-β1, it is not surprising that in our study intense staining of TGF-β1 in the tubular epithelium coexisted with intense peritubular expression of α-SMA.

In the renal medulla, we observed a higher TGF-β1 expression in the tubules and vessels from the Ang-Na groups, except in the Ang–Na 1.5 group, where TGF-β1 expression was significantly decreased.

Interestingly, Ang II increased NF-κB expression independently of hemodynamic changes, GFR or the sodium concentration infused.

Ang II is involved in tubulointerstitial fibrosis *via* its non-hemodynamic-effects on renal function. Ang II stimulates cellular hypertrophy of proximal tubular cells dependent on TGF-β1 expression. In contrast to its effects on proximal tubular cells, Ang II stimulates proliferation and increase of TGF-β1 mRNA expression in rat glomeruli endothelial cells and of more distal nephron segments, such as cells isolated from the thick ascending limb of Henle's loop. Among the downstream targets of TGF-β1 is hSGK1, a volume regulated serine/threonine kinase cell. TGF-β1 upregulates hSGK1, which leads to marked stimulation of the epithelial sodium channel ENaC and the Na/K/2Cl cotransporter (*16*). The enhanced expression of hSGK1 could favor renal sodium retention, enhanced energy consumption and hyperfiltration. Any increase of cell volume mediated by hSGK1-induced Na entry would impair matrix protein degradation and thus enhance the net deposition of matrix protein. In addition, Ang II could induce apoptosis of renal cells under certain conditions. Taking all these factors together, it could be suggested that the observation of a decrease in TGF-β1 expression in the medulla but not the cortex in the Ang–Na 1.5 groups was attributable to an adaptive process to protect the renal medulla from sodium overload.

In summary, Ang II doses to low to induce hypertension exacerbated intrarenal TGF-β1, α-SMA and NF-κB expression response to acute sodium overload, even before any increase in arterial pressure or development of glomerular functional alterations. These findings demonstrate the presence of a close relationship between Ang II–salt excess and renal inflammation and fibrosis. Moreover, this interaction could stimulate the expression of profibrogenic cytokines independently of BP levels.

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