Renovascular remodeling and renal injury after extended angiotensin II infusion

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Abstract

Chronic angiotensin II (Ang II) infusion for one or two weeks leads to progressive hypertension and induces inward hypertrophic remodeling in preglomerular vessels, which is associated with increased renal vascular resistance (RVR) and decreased glomerular perfusion. Considering the ability of preglomerular vessels to exhibit adaptive responses, the present study was performed to evaluate glomerular perfusion and renal function after six weeks of Ang II infusion. To address this study, male Wistar rats were submitted to fictitious surgery (control) or osmotic minipump insertion (Ang II 200 ng/kg/min, 42 days). A group of animals were treated or co-treated with losartan (10 mg/kg/day), an AT1 receptor antagonist, between the 28th and 42nd day. Chronic Ang II infusion increased systolic blood pressure to 185 ± 4 mmHg compared with 108 ± 2 mmHg in control rats. Concomitantly, Ang II-induced hypertension increased intrarenal Ang II level and consequently, preglomerular and glomerular injury. Under this condition, Ang II enhanced the total renal plasma flow (RPF), glomerular filtration rate (GFR), urine flow and induced pressure natriuresis. These changes were accompanied by lower RVR and enlargement of the lumen of interlobular arteries and afferent arterioles, consistent with impairment of renal autoregulatory capability and outward preglomerular remodeling. The glomerular injury culminated with podocyte effacement, albuminuria, tubulointerstitial macrophage infiltration and intrarenal extracellular matrix accumulation. Losartan attenuated most of the effects of Ang II. Our findings provide new information regarding the contribution of Ang II infusion over two weeks to renal hemodynamics and function via AT1 receptor.
Introduction

Hypertension is a recognized cause of chronic kidney disease (CKD) and end-stage renal disease (ESRD). Sustained hypertension with high plasma Ang II levels is associated with vascular remodeling in both intra and extrarenal vasculature characterized by cellular hypertrophy and narrowing of the lumen of resistance vessels (45). In addition, in the Ang II-infused rat model, sustained elevations in circulating Ang II induce a progressive increase of intrarenal renin-angiotensin system (33, 34, 39, 61). Under this condition, intrarenal Ang II interacts mainly with the AT1 receptors to sustain the hypertension, but it also induces progressive increases in reactive oxygen species (ROS), inflammation and vascular fibrosis due to accumulation of extracellular matrix (ECM) material and collagen types I, III and IV in the resistance vessels, glomeruli and renal interstitium (20, 32, 40, 57). Indeed, at the kidney level, Ang II-induced inward hypertrophic remodeling, particularly in preglomerular vessels, can increase renal vascular resistance (RVR) and decrease renal blood flow (RBF) and glomerular filtration rate (GFR), as extensively described by many research groups (18, 36, 52, 60). However, most of these studies of renovascular remodeling were performed in animals after one or two weeks of Ang II exposure.

It is known that hypertension is an important cause of the renal autoregulation decline (27). In addition, sustained elevations of intrarenal Ang II combined with ROS biodisposinibility also contribute to loss of renal autoregulatory efficiency leading to renal injury (8, 20, 26, 40). Indeed, several studies reported that Ang II infusion for one or two weeks not only impairs the autoregulatory response of the interlobular arteries and afferent arterioles, but also attenuates afferent arteriolar responses to adenosine triphosphate (ATP) and P2X; receptor agonist (10, 25, 28, 29, 59).
In line with these facts, the ability of preglomerular vessels to exhibit adaptive responses, opens the question of what could happen if the infusion of Ang II is extended for over than two weeks. Would the preglomerular injury associated with inward hypertrophic remodeling become irreversible? Thus, we hypothesized that Ang II-induced hypertension for six weeks impairing preglomerular autoregulation would induce significant changes in glomerular perfusion combined with renal injury. Using a six-week Ang II-infused model co-treated or not with losartan, an AT1 receptor antagonist, we aimed to determine the following: 1) whether chronic Ang II-induced hypertension is related to changes in renal hemodynamics associated with adaptative responses of preglomerular vessels (interlobular arteries and afferent arterioles); 2) the potential factors related to glomerular and tubulointerstitial injury in this condition and 3) the contribution of chronic Ang II infusion to AT1-mediated intrarenal Ang II synthesis. In the current study, we demonstrated that chronic Ang II infusion in rats induces persistent hypertension associated with intrarenal Ang II synthesis and severe changes in glomerular function via AT1 receptor. In addition, we provide the first report of enlargement of the preglomerular vessels accompanied by increases in RBF and GFR in chronic Ang II-induced hypertension, suggesting an impairment of renal autoregulatory capability. Furthermore, we demonstrated that preglomerular, glomerular and tubulointerstitial injuries are associated with inflammatory processes, increases in extracellular matrix (ECM) accumulation and albuminuria.

**Material and Methods**

*Animals and study design.* All procedures and protocols used in this study were approved by the Institutional Animal Care and Use Committee of University of São Paulo (Protocol no. 139, pages 110/02). Male Wistar rats (n = 80, weighing 160-200g) were obtained from the animal care facility of the Department of Physiology and
Biophysics, Institute of Biomedical Sciences, University of São Paulo, Brazil. All animals were housed at the department facility under standard conditions (constant temperature of 22 °C, 12:12-h light-dark cycle and 60% relative humidity). The rats were fed standard rat chow and provided water *ad libitum*. They were randomly allocated into the following four groups (n = 20 per group): control rats (with fictitious surgery), losartan treated rats, Ang II-infused rats (42 days - six weeks) or Ang II and losartan-treated rats (42 and 14 days, respectively). The rats were anesthetized with ketamine (75 mg/kg, i.p.) and xylazine (4 mg/kg, i.p.) (Virbac, Jurubatuba, SP, BR) and a dorsal midline incision was made to create a subcutaneous (s.c.) pocket, where the osmotic minipump (model 2006, Alzet Osmotic Pumps Company, Cupertino, CA, USA) containing Ang II (200 ng/kg/min/42 days, Tocris Bioscience, Bristol, UK) was inserted. The control group was also maintained under observation for the same period after fictitious surgery. A group of rats named as Ang II/losartan, was co-treated with losartan (10 mg/kg/day, s.c., DuPont 753, Merck Pharmaceuticals, Deepwater, NJ, USA) between the 28th and 42nd days after Ang II minipump insertion. At the present study, a group of rats was treated only with losartan and used as a control for the Ang II/losartan group. The rats were individually housed for food and water intake as well as body weight (BW) evaluation. Body weight gain was calculated using the following equation: (final BW - initial BW)/6 weeks.

**Blood pressure measurements.** Tail-cuff blood pressure was evaluated weekly by noninvasive tail-cuff plethysmography (Panlab/Harvard Apparatus, Barcelona, SP). The rats were acclimatized to a blood pressure instrument for 20 min before the readings were obtained to ensure that the measurements were accurate. The tail pulse was detected by passing the tail through a tail-cuff sensor attached to an amplifier. The average tail-cuff blood pressure was subsequently obtained from eight sequential cuff
inflation-deflation cycles. At the end of the treatment, direct blood pressure was also measured in conscious animals (n = 6 per group). For this purpose, one day before the experiments, the rats were anesthetized with ketamine (75 mg/kg, i.p.) and xylazine (4 mg/kg, i.p.) (Virbac), and a polyethylene catheter (PE-50 filled with heparinized saline; Clay Adams, Franklin Lakes, NJ, USA) was introduced into the right carotid artery and exteriorized in the mid-scapular region. Arterial pressure and heart rate were measured with a pressure transducer (model DT-100; Utah Medical Products, Midvale, UT, USA) and these values were registered using an interface and data acquisition software (Power Lab 4/25; AD Instruments, Sydney, Australia, AU); 1 kHz sampling rate), as previously described (17). Heart rate was determined from the intrabeat intervals.

A separate group of animals (n = 10 per group) was used for determinations of the plasma and intrarenal hormone concentrations and albuminuria. For these assessments, at twenty-four hours prior to the determinations, the control and treated rats were individually placed into metabolic cages for the monitoring of urine output. Urine samples were collected and used to determine the urinary albumin excretion. The rats from metabolic cages were anesthetized with Zoletil® (50 mg/kg zolazepam and 50 mg/kg tiletamine) and Virbaxyl® (5 mg/kg xylazine) (Virbac) and a polyethylene catheter (PE-50 filled with heparinized saline; Clay Adams, Franklin Lakes, NJ, USA) was introduced into the aorta artery and approximately 4mL of blood were collected for hormone measurements. The kidneys were immediately perfused with a solution containing 0.1M sodium phosphate buffer, 0.34M sucrose, 0.3 M NaCl (pH 7.2), frozen in liquid nitrogen and stored at -80°C.

Plasma and intrarenal angiotensin measurements. The plasma and intrarenal angiotensin levels were measured by reversed-phase high-performance liquid chromatography (HPLC), as previously described (13).
**Albuminuria.** The urinary albumin concentration was determined with an ELISA kit specific for rat urine albumin (Nephrat Kit; Exocell, Philadelphia, PA). The experiments were carried out following the manufacturer's instructions.

**Renal function evaluation.** After the blood pressure measurements, the animals were anesthetized with Zoletil® (50 mg/kg zolazepam and 50 mg/kg tiletamine) and Virbaxyl® (5 mg/kg xylazine) (Virbac), and placed on a warm table to maintain body temperature and tracheostomized using PE-260 tube to maintain ventilation. As previously described (50) and summarized here, the right carotid artery and right jugular vein were cannulated using PE-50 catheter (Clay Adams) for blood sample collection and continuous fluid infusion, respectively. Prior to clearance experiments, the first urine sample (approximately 1mL) was collected directly from the urinary bladder of each animal for further analysis of osmolality and determination of electrolyte content and podocyte count. A blood sample was also collected for osmolality and electrolyte analyses. Then, renal hemodynamic and function measurements were performed over a period of 3h by the following steps: 1) infusion of a control solution (0.9 NaCl plus 3% mannitol - to ensure urine production during clearance experiment) for 30min (0.1mL/min) using an infusion pump (Harvard Instruments, Holliston, MA, USA); 2) infusion of a 1mL bolus containing 300mg/kg inulin (Sigma Aldrich, St. Louis, MO, USA) and 7mg/kg sodium para-aminohippurate (PAH, Sigma Aldrich); and 3) 30min of continuous infusion with a solution containing 0.9% NaCl, 3% mannitol, 5mg/kg inulin and 1mg/kg PAH at 0.1mL/min (Sigma Aldrich). After these initial steps, four subsequent clearance periods were performed with the same infusion solution and rate. Each period consisted of simultaneous urine and arterial blood collection at the end of the 30min infusion. RPF and GFR were calculated according to PAH and inulin clearance, respectively. The filtration fraction (FF) was obtained as the GFR/RPF ratio.
and expressed as a percentage (%). RBF was estimated as RPF/(1-hematocrit) and RVR was calculated using the mean arterial pressure (MAP) to RBF ratio. The plasmatic and urinary sodium levels were measured using flame photometry (Roche, Auckland, New Zealand, NZ) and osmolality was measured using an osmometer (Precision Systems, Inc., Natick, MA, USA). Clearance (C) was calculated using the following formula, where x is the urine or plasma concentration of a substance and V represents urine flow rate: C = (Urine$\_x \cdot V$)/Plasma$\_x$.

**Urinary podocyte effacement.** Approximately 800µL of urine from the urinary bladder of each animal were used for podocyte effacement studies. The urine sample was centrifuged and washed with PBS, and cells were then seeded into a six-well plate containing collagen type I and Dulbecco's modified Eagle's medium (DMEM). Differentiated podocyte cultures, which do not proliferate, were evaluated for approximately 10 days. Then, the podocyte population in each well was counted using an inverted microscope (Nikon, Tokyo, JP) and the number of podocytes per well was compared between control and treated groups.

**Renal morphology.** Upon completion of the clearance experiments, the kidneys were immediately perfused with 20mL/min PBS (10mM sodium phosphate buffer containing 0.15M NaCl, pH 7.4) at room temperature through the abdominal aorta using a peristaltic perfusion pump (Milan Scientific Equipment, Curitiba, PA, BR). One kidney per rat was isolated, removed, weighed and used for quantitative PCR (qPCR). The remaining kidney was fixed in 4% paraformaldehyde solution, removed, dehydrated, and embedded in paraffin for morphological assessments. For these assays, 4µm thick kidney sections were stained using the Verhoeff method (to identify elastic fibers and collagen) or the periodic acid-Schiff (PAS) method (to evaluate glomerulosclerosis). The renal morphology was evaluated blindly by two independent
persons by using a light microscope (Eclipse 80i, Nikon). Approximately 6 interlobular arteries and 15 afferent arterioles from the renal cortical area per rat were included in the analysis. The luminal radius \( r = \text{diameter}/2 \) and media to lumen area ratio \( (M/L) \) were calculated. For glomerular dimensional and glomerulosclerosis analysis, all glomeruli with apparent macule dense and afferent arterioles from the renal cortical area of each rat were included. The area of each glomerulus was determined and the mean glomeruli areas were obtained using a computerized morphometry program (NIS-Elements D, Nikon). Glomerulosclerosis was graded using PAS-stained kidney sections according to the scoring system described by Saito et al (46). For each tissue section, the glomeruli were graded as follows: Grade 0, normal; Grade 1, sclerotic area of up to 25% (minimal); Grade 2, sclerotic area of 26-50% (moderate); Grade 3, sclerotic area of 51-75% (moderate to severe); and Grade 4, sclerotic area >75% (severe). The glomerulosclerotic area index (GSI) was then calculated using the following formula,

\[
\text{GSI} = \frac{(1 \times \text{NG1}) + (2 \times \text{NG2}) + (3 \times \text{NG3}) + (4 \times \text{NG4})}{(\text{NG1} + \text{NG2} + \text{NG3} + \text{NG4})}
\]

**Immunohistochemical staining.** As previously described (48) and summarized here, kidney sections (4µm thick) were deparaffinized and incubated with a primary antibody rabbit anti-desmin (1:500, Abcam, Cambridge, UK); or mouse anti-ED1 (CD68, 1:50, Dako, San Diego, CA, USA) for 60 min at room temperature. Nonspecific protein binding was blocked by incubation with 10% goat serum in PBS for 60 min. The reaction products were detected using the avidin-biotin-peroxidase method. The sections were counterstained with methyl green, dehydrated and mounted. Immunostained proteins were analyzed blindly by one independent person using a computerized morphometry program (NIS-Elements, Nikon). The anti-CD68 antibody reacts to a cytoplasmic antigen present in monocytes and macrophages and the mean
number of ED1-positive cells (macrophages) was obtained by calculating the mean counts per field and was compared between the control and treated groups. Desmin expression can be used as a reliable marker of glomerular damage and its staining was qualitatively analyzed.

**Intrarenal mRNA expression.** As previously described (50) and summarized here, total kidney RNA was obtained using TRIzol LS Reagent (Life Technologies, Carlsbad, CA, USA) and a RNA extraction kit (Qiagen Sciences, Germantown, MD, USA). Then, 2 μg total RNA was reverse transcribed using random hexamers (High-Capacity cDNA Reverse Transcription Kit; Life Technologies) and real-time PCR was performed using a StepOnePlus (Life Technologies) machine and TaqMan assay system (Life Technologies). The following TaqMan probes were used: renin (Ren), Rn00561847_m1; AT1 receptor (Agtr1a), Rn02758772_sl; AT2 receptor(Agtr2), Rn00560677_sl; tumor necrosis factor α (Tnfa), Rn99999017_m1; transforming growth factor beta (Tgfb), Rn00579674_ml; collagen III (Col3a1), Rn01437681_m1; integrin β1 (Itgb1), Rn00566727_ml; and GAPDH (Gapdh), Rn01775763_g1. All qPCRs were performed using 20ng cDNA, and all samples were assayed in duplicate. The comparative cycle threshold \( (2^{-ΔΔCt}) \) method was used for data analysis. The data were normalized to GAPDH expression and expressed as a fold change relative to the control group.

**Statistical analysis.** The results were evaluated using Student’s t-test for comparisons between two groups and one-way ANOVA with the Bonferroni correction to detect differences between three or more groups with a normal distribution using GraphPad Prism Software (GraphPad Software, Inc, San Diego, CA, USA). The results are expressed as the mean ± standard error of the mean (SEM) and p<0.05 was considered significant.
Results

Physiological parameters. As shown in Table 1, food and water intake, final body and kidney weights as well as body weight gain were similar in all groups. Although the water intake has increased by 22% in Ang II group, it did not differ statistically from the control rats.

Blood pressure. As shown in Figure 1 A and Table 1, the systolic blood pressure (SBP), measured in conscious animals using tail-cuff plethysmography, was similar in all animals at the onset of the study (0 week). The SBP progressively increased in the chronic Ang II-infused rats as compared to values observed in the control rats. After the 3rd week of Ang II treatment, SBP values reached a plateau, which remained stable until the 6th week. The losartan treatment alone did not modify this parameter as compared to control group. However, losartan co-treatment significantly reduced SBP in Ang II-infused rats; however it was still increased compared to control rats. In addition, the mean arterial pressure (MAP), directly measured in conscious animals, reinforce the tail cuff results, where we can observe that the chronic Ang II-infused rats had a higher average MAP than control rats at the end of the treatment, and losartan reduced the MAP in Ang II-infused rats to the control levels (Figure 1 B).

Ang II plasma level and intrarenal RAS components. As shown in Figure 2 A, in the Ang II-infused rats, the plasma Ang II level was significantly increased compared with the controls. The Ang II plasma levels in losartan alone or Ang II/losartan co-treated rats are similar to those observed in the controls rats. In addition, chronic Ang II infusion did not change AT1 or AT2 intrarenal mRNA expression (Table 1) or intrarenal renin mRNA expression as shown in Figure 2 B. However, the renin mRNA
expression was increased in losartan treated group, but restored to the controls levels in
the Ang II/losartan co-treated rats. In addition, the intrarenal Ang I and Ang II levels
were significantly increased in the Ang II-infused rats compared with the respective
controls. Losartan alone did not change the intrarenal Ang I and Ang II levels values
and Ang II/losartan co-treatment restored this parameter to control levels (Figure 2 C
and D).

Renal hemodynamics and tubular function. Six-weeks of Ang II-treatment
significantly reduced RVR (Figure 3 A), did not change the hematocrit, but increased
RBF (Table 1) and RPF and GFR (Figure 3 B and C) compared to control rats.
However, the FF (GFR/RPF ratio) was decreased in Ang II-infused rats compared to the
controls (Figure 3 D). Losartan alone did not change those parameters; however, the
treatment of Ang II-infused rats with losartan did not change the RVR observed in
Ang II-infused rats, but it was able to restore the other parameters to those observed in
the controls rats. Urine flow rate (Figure 3 E) was increased in the Ang II-infused rats
compared with the controls. Losartan alone did not change this parameter and the co-
treatment of Ang II-infused rats with losartan restores this parameter to the control
levels.

Na\(^+\) in plasma and urine and plasma osmolality. As shown in Table 1, neither
Ang II infusion nor losartan alone or the co-treatment with losartan changed the Na\(^+\)
plasma concentration or plasma osmolality. The filtered and excreted Na\(^+\) loads were
increased in the Ang II-infused rats, remained unchanged in losartan treated rats and
were recovered in the Ang II-infused rats co-treated with losartan compared to the
respective control rats. However, the urinary sodium level (U\(_{Na^+}\)) and fractional
excretion of Na\(^+\) remained unchanged between the groups.
Renal morphology. Renal cortical morphologic analysis of the Ang II-infused rats revealed injury throughout the cortical area, including perivascular and tubulointerstitial fibrosis, consistent with structural and functional changes. As shown in Figure 4 A - B, in the Ang II-infused rats, the interlobular arteries (indicated by arrows) displayed significantly increased lumen radius compared to those of the control rats. Losartan alone did not change the interlobular arteries structure and losartan co-treatment in Ang II-infused rats restored this parameter to the control condition. The interlobular media/lumen (M/L) ratio, an index of arterial resistance, was also calculated, and it was decreased in the Ang II-infused rats compared to the controls, unchanged in losartan treated and partially recovered in Ang II-infused rats co-treated with losartan (Figure 4 C).

The same analysis was performed to assess the afferent arterioles, as shown in Figure 5 A. As described by interlobular arteries, in the Ang II-infused rats, the afferent arterioles (indicated by arrows) exhibited significantly increased lumen radius compared to those of the control rats, remained unchanged in losartan treated group and losartan co-treatment in Ang II-infused rats reduced the lumen radius of afferent arterioles to control levels (Figure 5 B). However, the afferent arteriole media/lumen (M/L) ratio remained unchanged among groups (Figure 5 C).

As shown in Figure 5 A and D, the glomerular area was significantly increased in the chronic Ang II-infused rats compared to the control rats, it remained unchanged in losartan treated group and the co-treatment with losartan in Ang II-infused rats was able to restore the glomerular area. Chronic Ang II infusion also induced glomerulosclerosis (indicated by arrows, Figure 6 A and Table 1) compared with the controls. Losartan alone did not change and in the Ang II-infused rats co-treated with losartan this parameter was decreased but remained elevated compared to the controls.
The glomerulosclerosis indice (GSI) was calculated and as shown in Figure 6 B it reinforces the morphological alterations observed.

*Immunohistochemical staining of desmin.* As shown in Figure 7 A, glomerular injury in the chronic Ang II-infused rats was demonstrated by strong desmin staining (indicated by arrows) compared with the control rats. There are no changes in this parameter in losartan-treated rats, but losartan co-treatment in Ang II-infused rats restored it. In addition, chronic Ang II infusion induced podocyte effacement (Figure 7 B) and albuminuria (Figure 7 C) compared to the controls. Losartan alone did not change the control conditions and these parameters were restored in Ang II/losartan co-treated rats.

*Immunohistochemical staining of ED1.* The number of ED1-positive cells was evaluated by immunohistochemical staining. As shown in Figure 8 A, a significant number of infiltrating ED1-positive cells/field was observed in the tubulointerstitial areas of the Ang II-infused rats compared to the controls. Losartan alone did not change the control condition, but the Ang II/losartan co-treatment significantly reduced the number of ED-1 positive cells in the tubulointerstitial areas as compared with Ang II-infused rats; however, it was increased compared to control rats (Figure 8 B).

*Intrarenal mRNA expression of inflammatory and extracellular matrix components.* As described above, morphologic and immunoreactive analyses demonstrated that chronic Ang II infusion induced severe changes in the preglomerular vessels, glomerular area and tubulointerstitial compartment, suggesting renal inflammation and fibrosis. These processes are complex and involve proinflammatory cytokine synthesis and the secretion of many ECM components. Our results (Figure 9 A-D) demonstrated that chronic Ang II infusion induced significant increases in TNFα, which suggests inflammatory activity and also increased the mRNA expression of
ITGβ1, TGFβ1 and Coll III, which suggests ECM accumulation, compared to the respective controls. Losartan alone or Ang II/losartan co-treatment did not change these parameters compared to the respective controls.

**Discussion**

As previously reported, rats chronically infused with Ang II develop persistent hypertension, which can be prevented by AT1 receptor antagonists (50, 61). Consistent with these findings, we observed sustained AT1 receptor-mediated hypertension in six-weeks of Ang II-infused rats. Although losartan-induced hypotensive response has been reported previously (2, 56, 61), other studies in healthy subjects and in animals (7, 9, 35), showed that this AT1 receptor antagonist alone produces little effect on blood pressure. Consistent with these finding and our previous study (50), we observed that losartan (10 mg/kg/day) did not change systolic blood pressure compared to the control rats, but decreased the Ang II-induced hypertension and renal injury.

In an Ang II-infused model, a sustained increase in the plasma Ang II level has been demonstrated (33). The present study confirms this finding, and suggests that the increased plasma Ang II levels are, in part, associated with Ang II infusion. Surprisingly, losartan treatments did not change this parameter, compared to the control group. Although did not differ statistically, in Ang II/losartan co-treated rats plasma Ang II levels reached 34% above the control group. Our findings with losartan (10mg/kg/day, s.c.) for two weeks are in agreement with previous study of Goldberg and colleagues (19) in which hypertensive patients were treated with losartan 25mg/day for the same extent. However, it differs from the study of Zou and colleagues (61) in which they observed that Sprage-Dawley rats treated with losartan 30mg/kg/day (in the drinking water) for two weeks, exhibited increased plasma renin activity and Ang II
levels. The divergences between these findings are relevant and appear to be associated in part, with the dose and administration route.

It is known that chronic Ang II infusion induces intrarenal RAS activation (47). Our observations are in agreement with this finding because although six weeks of Ang II infusion did not change the intrarenal renin mRNA level, it induced significant increases in the intrarenal Ang I and Ang II levels. These results provide evidence of an intrarenal positive feedback loop, in which sustained plasma Ang II level induces de novo synthesis of intrarenal Ang I and Ang II. Although the exact mechanism has not yet been elucidated, Ang II is thought to induce TGFβ1 and NF-kappaB expression to promote intrarenal angiotensinogen synthesis (6). It has been proposed that high levels of proximal tubular angiotensinogen contribute to tubular Ang II syntheses (39, 47). In addition, it is known that under normal condition Ang II via the AT1 receptor, inhibits renin secretion (11) Thus, the enhanced intrarenal renin mRNA expression observed by us in the losartan group, results from the blockade of negative feedback mechanism of Ang II on the synthesis and secretion of renin by juxtaglomerular cells. Although Ang II did not alter intrarenal AT1 or AT2 mRNA expression in the present study, consistent with previous reports (23), our data demonstrated that AT1 receptor contributes to the internalization and/or synthesis of intrarenal Ang I and Ang II, since it was inhibited by losartan.

One or two weeks of Ang II infusion gradually increases blood pressure, oxidative stress, inflammation and fibrosis (20, 32, 40, 41, 57), all associated with the preglomerular inward hypertrophic remodeling which has been shown to be closely related to progressive increase in RVR and decrease in RBF and GFR (1, 18, 60). However, under this condition, the renovascular compensatory response seems to be associated with numerous factors as follows. In the decreased GFR resulting from
inward hypertrophic remodeling, less $\text{Na}^+$ is in the forming urine, and most will be reabsorbed before reaching the macula densa, which will result in decreased MD-TGF mechanism. In addition, the Ang II-attenuating $\text{P2X}_1$ receptor activity in afferent arterioles potentiates the disturbance in renal autoregulation (10, 25, 28, 29, 59). Ang II also reduces preglomerular myogenic response (30). Together those changes lead to progressive increase in glomerular perfusion (5, 21, 42). Our data with six weeks of Ang II infusion are consistent with those findings and support the concept that long term Ang II exposition combined with changes in blood pressure may impair renal autoregulatory capability, thus allowing significant decreases in RVR and increases in the RBF, RPF and GFR. Furthermore, we did not observe differences in RVR between the Ang II and Ang II/losartan groups, corroborating with the notion that in these conditions the kidneys were not able to autoregulate blood flow and glomerular filtration rate. On the other hand, considering that in the Ang II-infused rats, RPF increased by approximately 38% compared to the 24% increase in the GFR, the FF, calculated according to the GFR/RPF ratio, was decreased compared to controls and it was corrected by losartan.

On the other hand, the mechanisms that start the transition between the inward hypertrophic remodeling and the enlargement of the diameters of the preglomerular vessels are complex and were not explored in the current study. However, the relationship between hypertension and loss of elastin efficiency has been shown to contribute to vessel thickening (3). These alterations, together with the increase in collagen, are likely associated to vascular mechanical abnormalities in hypertension (3). Interestingly, the connective tissues of both the interlobular artery and afferent arteriole walls were notably increased in the chronic Ang II-infused rats, suggestive of alterations in ECM components, which has been implicated in the development and/or progression
of structural alterations (vascular remodeling) in other small vessels, as described by
Touyz and colleagues (51). Collectively, our morphological observations suggest an
Ang II-induced preglomerular outward remodeling, which was prevented by losartan.

Increased GFR is a critical determinant of glomerular injury and progressive
glomerulosclerosis (5, 21, 42). Capillary expansion and mesangial cell straining due to
high blood flow can stimulate mesangial cell proliferation and uncontrolled synthesis of
ECM components (14, 24). Our results are consistent with these findings because in the
Ang II-infused rats, the increased GFR was accompanied by increases in the glomerular
area and glomerulosclerosis. In addition, we also observed a pronounced increase in
glomerular desmin staining intensity. Desmin is an important marker of glomerular
injury and it may be associated with the observed glomerular hypertrophy. It is known
that in the healthy glomeruli, desmin is distributed mainly in mesangial cells. However,
desmin staining in rat podocytes can be a reliable marker of podocyte injury (16).
Indeed, analysis of the urinary cultures revealed an increase in the viable podocyte
number in the Ang II-infused rats. Nevertheless, podocyte injury seems to be associated
with glomerulosclerosis and proteinuria (54). In the present study, we suggest that the
podocyte loss induced by Ang II infusion may be associated with the observed
glomerulosclerosis and albuminuria. However, we also suggest an important
contribution of the stretching of glomerular capillaries, induced by high blood flow
and/or changes in the glomerular basement membrane (GBM), to the podocyte
effacement. All of the above mentioned effects of Ang II were restored by losartan,
indicating an important contribution of AT1 receptor to glomerular morphology and
hemodynamic changes.

The increased GFR observed in the Ang II-infused rats was accompanied by
increased urine flow rate. In addition, the filtered and excreted Na⁺ loads were increased
probably due to pressure natriuresis. The effects of Ang II on these parameters were mediated by AT1. It is known that in chronic Ang II-infused models, sodium excretion is complex and depends on the dose and duration of the treatment, as well as the magnitude of the blood pressure response (58). Usually, chronic Ang II infusion initially induces sodium retention, with restoration of the sodium balance occurring at an elevated arterial pressure (37). The results of the present study are consistent with these and other findings and suggest that the contribution of both the proximal tubule as the major site of filtered load absorption and distal nephron segments, which are responsible for the fine regulation of sodium excretion via sodium transporters (15, 44, 49), are important for maintenance of the sodium balance.

A large number of experimental studies have shown that local RAS activation is associated with several key events of the inflammatory processes (38) and ROS production (53, 55). Moreover, Ang II favors the recruitment of infiltrating inflammatory cells into tissues by stimulating the production of specific cytokine/chemokines (4). Consistent with these findings, our results suggest that Ang II induced severe inflammatory process, through the recruitment of macrophages (enhanced number of ED1-stained cells particularly in the interstitium). Furthermore, the Ang II infusion enhanced the TNFα mRNA expression. Macrophages are the major producers of TNFα and interestingly, they are also highly responsive to TNFα (43), which have critical role in the development of many chronic inflammatory diseases (12), regulating many cell functions, including cell proliferation, differentiation and apoptosis (43). These results provide evidence of active interactions between Ang II/AT1 and macrophages recruitment and TNFα synthesis in the evolution of renal injury.
Inflammation of the tubulointerstitial compartment leads to renal fibrosis, which is associated with ECM accumulation. The ECM is a protein complex composed of cell adhesion proteins, including collagen, fibronectin, laminin, and proteoglycans. Cell adhesion to the ECM is mainly mediated by members of the β1 integrin family expressed on the cell surface (31). β1 integrin is a receptor for many signaling pathways, and it can also function as a force sensor, transducing mechanical stimuli into biochemical signals (22). In the kidney, β1 integrin is responsible for glomerular cell-ECM adhesion, modulates renal fibrosis and tubular cell signaling (22, 31). Consistent with these findings, we observed that intrarenal β1 integrin, TGFβ1 and collagen III mRNA expression was significantly increased in the Ang II-infused rats and that losartan reduced the stimulatory effect of Ang II on these parameters.

In conclusion, we demonstrated that chronic Ang II infusion induced persistent hypertension via AT1 receptor, which was associated with an increase in the intrarenal Ang II level and consequently, severe preglomerular and glomerular injury. In addition, our results demonstrate for the first time that chronic Ang II infusion combined with hypertension, via AT1 receptor, induced enlargement of the preglomerular vessels accompanied by increases in RBF, GFR, urine flow and pressure natriuresis. Furthermore, our data suggest that most of the changes observed are due to impairment of renal autoregulatory capability. The glomerular injury induced by intrarenal Ang II/AT1 receptor culminated with podocyte effacement, albuminuria, tubulointerstitial macrophage infiltration and intrarenal accumulation of cytokines and ECM components. Taken together, our findings provide new information regarding the contribution of six-weeks Ang II infusion to renal hemodynamics and function via AT1 receptor. These findings might be important to consider in the treatment of chronic hypertensive patients with renovascular remodeling combined with glomerular injury.
Sources of Funding

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Conflict of interest statement: None

References


22. Hamzeh MT, Sridhara R, and Alexander LD. Cyclic stretch-induced TGF-β1 and fibronectin expression is mediated by β1-integrin through c-Src- and STAT3-


**Figure Legends**

**Figure 1**: The effects of Ang II treatment and/or losartan on systolic blood pressure (SBP) progression (A), as measured by tail-cuff plethysmography during treatment for six weeks (n = 20 per group, and values are mean of each group per week). The arrows indicate the beginning of treatment with Ang II and/or losartan. At the end of treatment, the direct arterial pressure was evaluated (n = 6 rats from each group), and the mean arterial pressure (MAP) was calculated (B). *p<0.001 vs. control; †p<0.001 vs. Ang II-infused rats.

**Figure 2**: The effects of Ang II treatment and/or losartan on the Ang II plasma level (A), intrarenal renin mRNA expression (B), and intrarenal Ang I (C) and Ang II (D) synthesis. The data are presented as the mean ± SE (n = 5-6 per group).

**Figure 3**: The effects of Ang II treatment and/or losartan on renal hemodynamics (n = 10 per group), including renal vascular resistance (RVR) (A), renal plasma flow (RPF) (B), glomerular filtration rate (GFR) (C), filtration fraction (FF) rate (D) and urine flow rate (E).
Figure 4: Representative photomicrographs of the renal cortex, illustrating morphological changes in interlobular arteries (IAs, indicated by arrows) from control rats, Ang II-infused rats, losartan treated rats and Ang II-infused rats co-treated with losartan (A). Kidney sections (4µm thick) were stained using the Verhoeff method, which stains elastic fibers (black) and collagen (magenta). Standard images were captured using a morphometric program (NIS-Elements) with a 20x objective, and highlighted images were captured with a 40x objective (magnifications of 200x and 400x, respectively). The IA lumen radius were determined \( r = \text{diameter}/2 \) (B), and the media/lumen ratios were calculated (C). Data obtained from 6 IAs per rat \((n = 10 \text{ per group})\) are presented as the mean ± SE. G, glomeruli; Los, losartan; bar = 50µm.

Figure 5: Representative photomicrographs of the renal cortex, illustrating morphological changes in afferent arterioles (AAs, indicated by arrows) from control rats, Ang II-infused rats, losartan treated rats and Ang II-infused rats co-treated with losartan (A). Kidney sections (4µm thick) were stained using the Verhoeff method. Standard images were captured using a morphometric program (NIS-Elements) with a 20x objective, and highlighted images were captured with a 40x objective (magnifications of 200x and 400x, respectively). The AA lumen radius were determined \( r = \text{diameter}/2 \) (B), and the media/lumen ratios were also calculated (C). Data obtained from 15 AAs per rat \((n = 10 \text{ per group})\) are presented as the mean ± SE. Using the same photomicrographs of the renal cortex, the outer glomerular edges were traced manually on a video screen, and the glomerular areas were calculated by using the software (NIS-Elements, Nikon). Glomerular area are presented as the mean ± SE (D), \((n = 10 \text{ per group})\). G, glomeruli; Los, losartan; bar = 50µm.

Figure 6: As illustrated by representative photomicrographs of the renal cortex, glomerulosclerosis (indicated by arrows) was analyzed in kidney sections (4µm thick)
stained with the periodic acid-Schiff (PAS) method from control rats, Ang II-infused rats, losartan treated rats and Ang II-infused rats co-treated with losartan (A). Standard images were captured using a morphometric program (NIS-Elements) with a 20x objective, and highlighted images were captured with a 40x objective (magnifications of 200x and 400x, respectively). For glomerulosclerosis evaluation, a scoring system was used as follows: Grade 0, normal; Grade 1, sclerotic area of up to 25% (minimal); Grade 2, sclerotic area of 25-50% (moderate); Grade 3, sclerotic area of 50-75% (moderate to severe; and Grade 4, sclerotic area >75% (severe). Values were obtained using software (NIS-Elements, Nikon). The glomerulosclerotic area index (GSI) was then calculated using the following formula, where NGx is the number of glomeruli with each given score for area: 

\[
GSI = \frac{[(1 \times NG1) + (2 \times NG2) + (3 \times NG3) + (4 \times NG4)]}{NG1 + NG2 + NG3 + NG4}.
\]

The quantitative data (n = 5/group) are expressed as the mean ± SE (B).

Figure 7: Representative photomicrographs of the renal cortex, illustrating changes in glomerular desmin expression (indicated by arrows). Kidney sections (4µm thick, n = 6 per group) from control rats, Ang II-infused rats, losartan treated rats and Ang II-infused rats co-treated with losartan (A) were immunohistochemically stained using a rabbit anti-desmin antibody. Standard images were captured using a morphometric program (NIS-Elements) with a 20x objective, and highlighted images were captured with a 40x objective (magnifications of 200x and 400x, respectively). Podocyte effacement presented as number of podocytes/well of urinary sample from control rats. Ang II-infused rats and Ang II-infused rats co-treated with losartan. Then, the number of attached podocytes in each well was determined. The data are presented as the mean ± SE (B). Albumin excretion in the control rats and Ang II-infused rats treated with
losartan (C). The data are presented as the mean ± SE (n = 9 - 10 per group). Ang II-infused rats; G, glomeruli; bar = 50µm.

Figure 8: Representative photomicrographs of the renal cortex, illustrating tubulointerstitial ED1-positive cells (indicated by arrows). Kidney sections (4µm thick, n = 10 per group) from the control rats, Ang II-infused rats, losartan treated rats and Ang II-infused rats co-treated with losartan (A) were immunohistochemically stained using a rabbit anti-ED1 antibody. Images were captured using a morphometry program (NIS-Elements) with a 20x objective, and highlighted images were captured with a 40x objective (magnifications of 200x and 400x, respectively). ED1-positive cells (macrophages) in the tubulointerstitial areas were counted, and the data are presented as the mean ± SE of 60 areas (B); bar = 50µm. The asterisks indicate only the highlighted areas.

Figure 9: Quantitative PCR analysis of renal tissues from the control rats and the rats treated with Ang II and/or losartan. TNFα (A), integrin (ITG) β1 (B), TGFβ1 (C) and collagen type III (Coll III) (D) mRNA expression levels. The data are presented as the mean ± SE (n = 5-7 per group) and all samples were assayed in duplicate.
Figure 1
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Figure 2
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Figure 3
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Figure 4
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Figure 5
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Figure 6
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Figure 8
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Figure 9
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Table 1: Differences among experimental groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ctl (n = 20)</th>
<th>AII (n = 20)</th>
<th>Los (n = 20)</th>
<th>AII/Los (n = 20)</th>
</tr>
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<tbody>
<tr>
<td>Food intake, g/day</td>
<td>18.4 ± 0.8</td>
<td>18.5 ± 1.3</td>
<td>17.6 ± 1.0</td>
<td>15.9 ± 1.1</td>
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<tr>
<td>Water intake, mL/day</td>
<td>35.0 ± 2.8</td>
<td>43.0 ± 3.8</td>
<td>35.1 ± 3.6</td>
<td>38.4 ± 4.4</td>
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<tr>
<td>Body weight gain, g/wk</td>
<td>33.5 ± 0.5</td>
<td>30.1 ± 1.3</td>
<td>30.7 ± 1.6</td>
<td>32.9 ± 1.2</td>
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<tr>
<td>Final body weight, g</td>
<td>358 ± 7</td>
<td>345 ± 13</td>
<td>363 ± 9</td>
<td>368 ± 10</td>
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<tr>
<td>Kidney weight/body weight, mg/g</td>
<td>4.11 ± 0.1</td>
<td>3.96 ± 0.3</td>
<td>4.31 ± 0.5</td>
<td>4.02 ± 0.5</td>
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<td>Initial SBP, mmHg</td>
<td>101 ± 1</td>
<td>106 ± 2</td>
<td>100 ± 3</td>
<td>109 ± 1</td>
</tr>
<tr>
<td>Final SBP, mmHg</td>
<td>108 ± 2</td>
<td>185 ± 4*</td>
<td>104 ± 3</td>
<td>125 ± 3*</td>
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<tr>
<td>Plasma Na⁺, mEq/L</td>
<td>142 ± 1</td>
<td>144 ± 2</td>
<td>139 ± 1</td>
<td>139 ± 1</td>
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<tr>
<td>Plasma osmolality, mOsmol/kg H₂O</td>
<td>304 ± 16</td>
<td>308 ± 13</td>
<td>298 ± 10</td>
<td>299 ± 7</td>
</tr>
<tr>
<td>Filtered load of Na⁺, mEq/min</td>
<td>1065 ± 38</td>
<td>1356 ± 40*</td>
<td>1180 ± 110</td>
<td>1081 ± 75$</td>
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<tr>
<td>Excreted load (U Na⁺ .V), mEq/min</td>
<td>1.68 ± 0.17</td>
<td>2.78 ± 0.23*</td>
<td>1.53 ± 0.11</td>
<td>1.28 ± 0.13*</td>
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<tr>
<td>U Na⁺, mEq/min</td>
<td>29.86 ± 4.8</td>
<td>38.50 ± 5.8</td>
<td>30.10 ± 4.3</td>
<td>24.25 ± 6.2</td>
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<tr>
<td>FE Na⁺ (%)</td>
<td>0.18 ± 0.02</td>
<td>0.21 ± 0.04</td>
<td>0.15 ± 0.03</td>
<td>0.14 ± 0.01</td>
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<tr>
<td>AT1 receptor mRNA (fold change)</td>
<td>1.01 ± 0.08</td>
<td>1.19 ± 0.20</td>
<td>-</td>
<td>-</td>
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<tr>
<td>AT2 receptor mRNA (fold change)</td>
<td>1.07 ± 0.50</td>
<td>0.98 ± 0.38</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>41 ± 2</td>
<td>46 ± 1</td>
<td>46 ± 3</td>
<td>46 ± 1</td>
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<td>Renal blood flow, mL/min</td>
<td>12.34 ± 0.20</td>
<td>18.85 ± 0.52*</td>
<td>12.22 ± 0.36</td>
<td>13.65 ± 0.31*</td>
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<tr>
<td>Normal glomeruli, %</td>
<td>35.5 ± 3.8</td>
<td>21.6 ± 2.3*</td>
<td>37.2 ± 2.9</td>
<td>23.2 ± 1.9¥</td>
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<tr>
<td>Glomerulosclerosis G1, %</td>
<td>33.4 ± 0.6</td>
<td>30.7 ± 0.9</td>
<td>35.6 ± 3.2</td>
<td>34.2 ± 1.4</td>
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<td>Glomerulosclerosis G2, %</td>
<td>18.0 ± 2.5</td>
<td>22.6 ± 2.1</td>
<td>14.3 ± 2.7</td>
<td>24.4 ± 1.7</td>
</tr>
<tr>
<td>Glomerulosclerosis G3, %</td>
<td>11.4 ± 1.9</td>
<td>15.8 ± 2.6</td>
<td>12.9 ± 0.5</td>
<td>14.2 ± 0.8</td>
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<tr>
<td>Glomerulosclerosis G4, %</td>
<td>1.7 ± 0.2</td>
<td>9.3 ± 2.2*</td>
<td>0.0 ± 0.0</td>
<td>4.0 ± 0.5$</td>
</tr>
</tbody>
</table>

Values are mean ± SE, number of animals (n) per group is indicated in parenthesis. *p<0.001 vs control (Ctl); ¥p<0.01 vs control (Ctl); ¥¥p<0.001 or ¥p<0.01 vs angiotensin II (AII) group. Los, losartan, SBP, systolic blood pressure; G1-G4, glomerulosclerosis grade.