Renovascular remodeling and renal injury after extended angiotensin II infusion

Fernando Augusto Malavazzi Casare,† Karina Thieme,‡ Juliana Martins Costa-Pessoa,† Luciana Venturini Rossoni,† Gisele Kruger Couto,† Fernanda Barrinha Fernandes,§ Dulce Elena Casarini,§ and Maria Oliveira-Souza†

†Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil; ‡Laboratory of Cellular and Molecular Endocrinology, Medical School, University of Sao Paulo, Sao Paulo, Brazil; and §Division of Nephrology, Department of Medicine, Federal University of Sao Paulo, Sao Paulo, Brazil

Submitted 16 October 2015; accepted in final form 5 March 2016

Abstract

Chronic angiotensin II (ANG II) infusion for 1 or 2 wk leads to progressive hypertrophy 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 6 wk of ANG II infusion. To address this study, male Wistar rats were submitted to sham surgery (control) or osmotic minipump insertion (ANG II 200 ng·kg⁻¹·min⁻¹·day⁻¹), an AT₁ receptor antagonist, between days 28 and 42. Chronic ANG II infusion increased systolic blood pressure to 185 ± 4 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 2 wk to renal hemodynamics and function via the AT₁ receptor.

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 in the intrarenal renin-angiotensin system (RAS) (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 1 or 2 wk 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 biodisponibility 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 1 or 2 wk not only impairs the autoregulatory response of the interlobular arteries and afferent arterioles but also attenuates afferent arteriolar responses to ATP and a P2X₁ 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 more than 2 wk. Would the preglomerular injury associated with inward hypertrophic remodeling become irreversible? Thus we hypothesized that ANG II-induced hypertension for 6 wk, impairing preglomerular autoregulation, would induce significant changes in glomerular perfusion combined with renal injury. Using a 6-wk ANG II-infused model cotreated 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 adaptive 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 the 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 ECM accumulation, and albuminuria.

MATERIALS 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 the University of São Paulo (protocol no. 110/02). Male Wistar rats (n = 80, weighing 160–200 g) were obtained from the animal care facility of the Department of Physiology and Biophysics, Institute of Biomedical Sciences, the University of São Paulo (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/group): control rats (with sham surgery), losartan-treated rats, ANG II-infused rats (42 days-6 wk), or ANG II- and losartan-treated rats (42 and 14 days, respectively). The rats were anesthetized with ketamine (75 mg/kg ip) and xylazine (4 mg/kg ip, Virbac, Juruatuba, São Paulo, Brazil), and a dorsal midline incision was made to create a subcutaneous (sc) pocket, where the osmotic minipump (model 2000; Alzet Osmotic Pumps, Cupertino, CA) containing ANG II (200 ng·kg⁻¹·min⁻¹, 42 days, Tocris Bioscience, Bristol, UK) was inserted. The control group was also maintained under observation for the same time period after sham surgery. A group of rats named as ANG II/losartan was cotreated with losartan (10 mg·kg⁻¹·day⁻¹ sc, DuPont 753, Merck Pharmaceuticals, Deepwater, NJ) between days 28 and 42 after ANG II minipump insertion. In 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 evaluation. Body weight gain and water intake as well as body weight evaluation. Body weight gain was calculated using the following equation: (final body weight – initial body weight)/6 wk.

Blood pressure measurements. Tail-cuff blood pressure was evaluated weekly by noninvasive tail-cuff plethysmography (Panlab/Harvard Apparatus, Barcelona, Spain). 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/group). For this purpose, 1 day before the experiments, the rats were anesthetized with ketamine (75 mg/kg ip) and xylazine (4 mg/kg ip, Virbac), and a polyethylene catheter (PE-50 filled with heparinized saline; Clay Adams, Franklin Lakes, NJ) was introduced into the right carotid artery and exteriorized in the midscapular region. Arterial pressure and heart rate were measured with a pressure transducer (model DT-100; Utah Medical Products, Midvale, UT), and these values were registered using an interface and data-acquisition software (1-kHz sampling rate, Power Lab 4/25; AD Instruments, Sydney, Australia), as previously described (17). Heart rate was determined from the intrabeat intervals.

A separate group of animals (n = 10/group) was used for determinations of the plasma and intrarenal hormone concentrations and albuminuria. For these assessments, at 24 h before 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 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), a polyethylene catheter (PE-50 filled with heparinized saline; Clay Adams) was introduced into the aortic artery, and ~4 ml of blood were collected for hormone measurements. The kidneys were immediately perfused with a solution containing 0.1 M sodium phosphate buffer, 0.34 M sucrose, and 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 reverse-phase 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), placed on a warm table to maintain body temperature, and tracheostomized using a PE-260 tube to maintain ventilation. As previously described (50) and summarized here, the right carotid artery and right jugular vein were cannulated using a PE-50 catheter (Clay Adams) for blood sample collection and continuous fluid infusion, respectively. Before clearance experiments, the first urine sample (~1 ml) was collected directly from the urinary bladder of each animal for further analysis of osmolality and determination of electrolyte content and podocyte number. A blood sample was also collected for osmolality and electrolyte analyses. Then, renal hemodynamic and function measurements were performed over a period of 3 h in the following steps: 1) infusion of a control solution (0.9 NaCl plus 3% mannitol to ensure urine production during clearance experiment) for 30 min (0.1 ml/min) using an infusion pump (Harvard Instruments, Holliston, MA); 2) infusion of a 1-mol bolus containing 300 mg/kg inulin (Sigma-Aldrich, St. Louis, MO) and 7 mg/kg sodium PAH (Sigma-Aldrich); and 3) 30 min of continuous infusion with a solution containing 0.9% NaCl, 3% mannitol, 5 mg/kg inulin, and 1 mg/kg PAH at 0.1 ml/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 urinary and arterial blood collection at the end of the 30-min 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, Australia), and osmolality was measured using an osmometer (Precision Systems, Natick, MA). Clearance was calculated using the following formula, where C is clearance, x is the urine or plasma concentration of a substance, and V represents urine flow rate: C = (urine·V)/plasma.

Urinary podocyte effacement. Approximately 800 µl 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 onto a six-well plate containing collagen type I and DMEM. Differentiated podocyte cultures, which do not proliferate, were evaluated for ~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 20 ml/min PBS (10 mM sodium phosphate buffer containing 0.15 M NaCl, pH 7.4) at room temperature through the abdominal aorta using a peristaltic perfusion pump (Milan Scientific Equipment, Curitiba, Brazil). 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 morphologic assessments. For these assays, 4-µm-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). 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 = diameter/2) and media-to-lumen area ratio (MLA) were calculated. For glomerular dimensional and glomerulosclerosis analysis, all glomeruli with apparent macula densa 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 glomerulosclerosis index (GSI) was then calculated using the following formula, where $N_G$ is the number of glomeruli with each grade:

$$GSI = \left( \frac{N_G}{NG0 + NG1 + NG2 + NG3 + NG4} \right) \times 100$$

**Immunohistochemical staining.** As previously described (48) and summarized here, kidney sections (4-μm 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) 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) and a RNA extraction kit (Qiagen Sciences, Germantown, MD). 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 (Agr1a), Rn02758772_sl; AT2 receptor (Agrr2), Rn00566077_sl; tumor necrosis factor-α (Tnfa), Rn99999017_m1; transforming growth factor-β (Tgfb), Rn00579674_ml; collagen III (Col3a1), Rn01437681_m1; integrin-β1 (Igb1), Rn00566727_ml; and GAPDH (Gapdh), Rn01775763_g1. All qPCRs were performed using 20 ng cDNA, and all samples were assayed in duplicate. The comparative cycle threshold ($2^{-\Delta\Delta C_{t}}$) method was used for data analysis. The data were normalized to GAPDH expression and expressed as the 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, San Diego, CA). The results are expressed as means ± SE, 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 the ANG II group, it did not differ statistically from the control rats.

Blood pressure. As shown in Fig. 1A 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 (week 0). The SBP progressively increased in the chronic ANG II-infused rats compared with values observed in the control rats. After week 3 of ANG II treatment, SBP values reached a plateau, which remained stable until week 6. Losartan treatment alone did not modify this parameter compared with the control group. However, losartan cotreatment significantly reduced SBP in ANG II-infused rats; however, it was still increased compared with control rats. In addition, MAP, directly measured in conscious animals, reinforces the tail-cuff results, where we observed that the chronic ANG II-infused rats had a higher average MAP than control rats at the end of the treatment, and losartan reduced MAP in ANG II-infused rats to control levels (Fig. 1B).

ANG II plasma level and intrarenal RAS components. As shown in Fig. 2A, 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-treated or ANG II/losartan-cotreated 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 Fig. 2B. However, renin mRNA expression was increased in the losartan-treated group but was restored to control levels in the ANG II losartan-cotreated rats. In addition, 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 intrarenal ANG I and ANG II level.
values, and ANG II/losartan cotreatment restored this parameter to control levels (Fig. 2, C and D).

Renal hemodynamics and tubular function. Six weeks of ANG II-treatment significantly reduced RVR (Fig. 3A), did not change the hematocrit, but increased RBF (Table 1) and RPF and GFR (Fig. 3, B and C, respectively) compared with control rats. However, the FF (GFR/RPF ratio) was decreased in ANG II-infused rats compared with the controls (Fig. 3D). Losartan alone did not change those parameters; however, the cotreatment 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. The urine flow rate (Fig. 3E) was increased in the ANG II-infused rats compared with the controls. Losartan alone did not change this parameter, and the cotreatment of ANG II-infused rats with losartan restored this parameter to the control levels.

Plasma Na\(^+\) and urine and plasma osmolality. As shown in Table 1, neither ANG II infusion nor losartan alone or the cotreatment 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 cotreated with losartan compared with the respective control rats. However, the urinary sodium level and fractional excretion of Na\(^+\) remained unchanged between the groups.

Renal morphology. Renal cortical morphological 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 Fig. 4, A and B, in the ANG II-infused rats, the interlobular arteries (indicated by arrows) displayed significantly increased lumen radius compared with those of the control rats. Losartan alone did not change the interlobular arterial structure, and losartan cotreatment in ANG II-infused rats restored this parameter to the control condition. The interlobular M/L ratio, an index of arterial resistance, was also calculated, and it was decreased in the ANG II-infused rats compared with the controls, unchanged in losartan-treated, and partially recovered in ANG II-infused rats cotreated with losartan (Fig. 4C).

The same analysis was performed to assess the afferent arterioles, as shown in Fig. 5A. As described for interlobular arterioles, in the ANG II-infused rats, the afferent arterioles (indicated by arrows) exhibited significantly an increased luminal radius compared with those of the control rats, remained unchanged in the losartan-treated group, and was reduced to control levels in the losartan/ANG II cotreatment group (Fig. 5B). However, the afferent arteriole M/L ratio remained unchanged among groups (Fig. 5C).

As shown in Fig. 5, A and D, the glomerular area was significantly increased in the chronic ANG II-infused rats compared with the control rats, it remained unchanged in the losartan-treated group, and the cotreatment with losartan in ANG II-infused rats was able to restore the glomerular area. Chronic ANG II infusion also induced glomerulosclerosis (indicated by arrows, Fig. 6A and Table 1) compared with the controls. Losartan alone did not change the control, and in the ANG II-infused rats cotreated with losartan this parameter was decreased but remained elevated compared with the controls. The GSI was calculated, and, as shown in Fig. 6B, it reinforces the morphological alterations observed.
Immunohistochemical staining of desmin. As shown in Fig. 7A, 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 cotreatment in ANG II-infused rats restored it. In addition, chronic ANG II infusion induced podocyte effacement (Fig. 7B) and albuminuria (Fig. 7C) compared with the controls. Losartan alone did not change the control conditions, and these parameters were restored in ANG II/losartan-cotreated rats.

Intrarenal mRNA expression of inflammatory and ECM components. As described above, morphological and immunohistochemical 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 (Fig. 9, A–D) demonstrated that chronic ANG II infusion induced significant increases in TNF-α, which suggests inflammatory activity and also increased the mRNA expression of integrin-β1, TGF-β1, and collagen type III, which suggests ECM accumulation, compared with the respective controls. Losartan alone or ANG II/losartan cotreatment did not change these parameters compared with 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 find-
ings, we observed sustained AT₁ receptor-mediated hypertension in rats infused with ANG II for 6 wk. Although the 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 findings and our previous study (50), we observed that losartan (10 mg·kg⁻¹·day⁻¹) did not change systolic blood pressure compared with 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 with the control group. Although not differing statistically, in ANG II/losartan co-treated rats plasma ANG II levels reached 34% above the control group. Our findings with losartan (10 mg·kg⁻¹·day⁻¹ sc) for 2 wk are in agreement with a previous study by Goldberg and colleagues (19), in which hypertensive patients were treated with losartan 25 mg/day for the same time period. However, that study differs from the study by Zou and colleagues (61), in which they observed that Sprague-Dawley rats treated with losartan 30 mg·kg⁻¹·day⁻¹ (in the drinking water) for 2 wk 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 6 wk of ANG II infusion did not change the intrarenal renin mRNA level, it induced significant
increases in intrarenal ANG I and ANG II levels. These results provide evidence of an intrarenal positive feedback loop, in which a 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 TGF-β1 and NF-κB expression to promote intrarenal angiotensinogen synthesis (6). It has been proposed that high levels of proximal tubular angiotensinogen contribute to tubular ANG II synthesis (39, 47). In addition, it is known that under normal conditions 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 the negative feedback mechanism of ANG II in 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 the 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 Na+/H+ is in the forming urine, and most will be reabsorbed before reaching the macula densa, which will result in the decreased macula densa-TGF mechanism. In addition, the ANG II-attenuating P2X1 receptor activity in afferent arterioles potentiates the disturbance in renal autoregulation (10, 25, 28, 29, 59). ANG II also reduces the preglomerular myogenic response (30). Together, those changes lead to a progressive increase in glomerular perfusion (5, 21, 42). Our data with 6 wk of ANG II infusion are consistent with those findings and support the concept that long-term ANG II exposure combined with...
changes in blood pressure may impair renal autoregulatory capability, thus allowing significant decreases in RVR and increases in 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 RBF and GFR. On the other hand, considering that in the ANG II-infused rats RPF increased by \( \frac{38}{100} \) compared with the 24% increase in the GFR, the FF, calculated according to the GFR/RPF ratio, was decreased compared with controls, and it was corrected by losartan.

On the other hand, the mechanisms that start the transition between 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 with 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. 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. Nev-

Fig. 7. A: representative photomicrographs of the renal cortex, illustrating changes in glomerular desmin expression (indicated by arrows). Kidney sections (4-µm thick, \( n = 6 \) /group) from control rats, ANG II-infused rats, losartan-treated rats, and ANG II-infused rats cotreated with losartan were immunohistochemically stained using a rabbit anti-desmin antibody. Standard images were captured using a morphometric program (NIS-Elements) with a \( \times 20 \) objective, and high-contrast images were captured with a \( \times 40 \) objective (magnifications of \( \times 200 \) and \( \times 400 \), respectively). Podocyte effacement presented as the number of podocytes/well of urinary samples from control rats, ANG II-infused rats, and ANG II-infused rats cotreated with losartan. B: the number of attached podocytes in each well was determined. Values are means ± SE. C: albumin excretion in the control rats and ANG II-infused rats treated with losartan. Values are means ± SE (\( n = 9 - 10 \) /group). Bar = 50 µm.
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 the 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 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 a severe inflammatory process, through the recruitment of macrophages (enhanced number of ED1-stained cells particularly in the interstitium). Furthermore, the ANG II infusion enhanced TNF-\(\alpha\) mRNA expression. Macrophages are the major producers of TNF-\(\alpha\) and, interestingly, they are also highly responsive to TNF-\(\alpha\) (43), which has a 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 macrophage recruitment and TNF-α 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 and modulates renal fibrosis and tubular cell signaling (22, 31). Consistent with these findings, we observed that intrarenal β1-integrin, TGF-β1, 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 the 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 the 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 6-wk ANG II infusion to renal hemodynamics and function via the AT1 receptor. These findings might be important to consider in the treatment of chronic hypertensive patients with renovascular remodeling combined with glomerular injury.

ACKNOWLEDGMENTS

The authors thank Dr. Adriana C. C. Girardi, Heart Institute (InCor), University of Sao Paulo Medical School, for kindly supplying Losartan (DuPont-Merck Pharmaceutical) and Dr. Rildo A. Volpini for assistance with the IHC experiments and analysis. The authors also thank Dr. Margarida de Mello Aires and Dr. Gerhard Malnic for providing some reagents used in this study.

GRANTS

This work was supported by the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP; grants 13/19569-3, 11/14022-0, and 13/23087-4) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; grant 302646/2012-4).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


AJP-Renal Physiol • doi:10.1152/ajprenal.00471.2015 • www.ajprenal.org


