Local Angiotensin II and Transforming Growth Factor-β1 in Renal Fibrosis of Rats

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Abstract—Studies have demonstrated that local angiotensin II (Ang II) generation is enhanced in repairing kidney and that ACE inhibition or AT₁ receptor blockade attenuates renal fibrosis. The localization of ACE and Ang II receptors and their relationship to collagen synthesis in the injured kidney, however, remain uncertain. Using a rat model of renal injury with subsequent fibrosis created with chronic elevations in circulating aldosterone (ALDO), we examined the distribution and binding density of ACE and Ang II receptors in repairing kidneys, as well as their anatomic relationship to transforming growth factor-β1 (TGF-β1) mRNA, type I collagen mRNA, collagen accumulation, and myofibroblasts. Two groups of animals (n=7 in each group) were studied: (1) normal rats served as controls, and (2) uninephrectomized rats received ALDO (0.75 μg/h SC) and 1% NaCl in drinking water for 6 weeks. Compared with control rats, in ALDO-treated rats we found (1) significantly (P<0.01) increased blood pressure, reduced plasma renin activity, and increased plasma creatinine levels, (2) diffuse fibrosis in both renal cortex and medulla, (3) abundant myofibroblasts at these sites of fibrosis, (4) significantly increased (P<0.01) binding density of ACE and Ang II receptors (60% AT₁, 40% AT₂) at the sites of fibrosis, and (5) markedly increased (P<0.01) expression of TGF-β1 and type I collagen mRNAs at these same sites. Thus, in this rat model of renal repair, the enhanced expression of ACE, Ang II receptors, and TGF-β1 is associated with renal fibrosis. Ang II generated at the sites of repair appears to have autocrine/paracrine functions in the regulation of renal fibrous tissue formation alone or through its stimulation of TGF-β1 synthesis.

Key Words: kidney ■ fibrosis ■ myofibroblasts ■ angiotensin II ■ growth substances ■ rats

Angiotensin (Ang) II, generated by endothelial and circulating ACE, is a classic endocrine hormone that plays a central role in the regulation of blood pressure and sodium homeostasis. There is accumulating evidence of Ang II generation by diverse cells in a variety of tissues. Such locally generated Ang II has autocrine/paracrine functions in the regulation of cell behavior and tissue structure. Local Ang II production is activated in certain pathological states in association with tissue repair; this includes injured blood vessel,1 injured kidney,2 and infarcted myocardium,3,4 where ACE and Ang II receptors are expressed by myofibroblasts (myoFb).5,6 The myoFb are phenotypically transformed fibroblast-like cells that express α-smooth muscle actin (α-SMA). They are rarely seen in normal tissue but appear with tissue repair, where they play a major role in fibrous tissue formation and wound contraction.7

A chronic inappropriate (relative to intravascular volume and sodium intake) activation of the circulating renin-angiotensin-aldosterone system (RAAS) as occurs in advanced heart failure is accompanied by progressive structural remodeling of the heart and systemic organs (eg, kidneys) by fibrous tissue. In the case of the kidney, such fibrous tissue accumulation is expressed as glomerulosclerosis and tubulointerstitial fibrosis.8,9 Regulatory mechanisms that are responsible for fibrous tissue formation in the kidneys in states of chronic hyperaldosteronism are of considerable interest. Many studies have demonstrated that ACE inhibitors or AT₁ receptor antagonists markedly attenuate renal fibrosis and preserve kidney function.10–12 However, it remains uncertain whether local Ang II is associated with fibrogenesis in the repairing kidney and contributes to the renoprotective effect of these pharmacological agents.

Mechanisms responsible for Ang II–promoting fibrous tissue formation are under investigation. In vitro studies have demonstrated that Ang II stimulates fibroblast collagen synthesis and expression of transforming growth factor-β1 (TGF-β1), an important mediator of fibrous tissue formation in repairing tissue. TGF-β1 is released as a latent form. While activated, TGF-β1 promotes fibroblast differentiation and proliferation, upregulates collagen synthesis, and inhibits collagenase release. The long-term administration of the AT₁ receptor antagonist losartan to rats significantly attenuates TGF-β1 synthesis and fibrosis in injured rat hearts and kidneys,13,14 suggesting that the role of Ang II in the promotion of fibrogenesis is achieved through stimulation of TGF-β1 synthesis and release via AT₁ receptors.
In the present study, we examined the expression and distribution of ACE and Ang II receptors in the repairing kidney and their anatomic relationship to renal fibrogenesis in a rat model of long-term ALDO infusion.

Methods

Animals
Eight-week-old male Sprague-Dawley rats were purchased from Harlan Sprague-Dawley. Two animal groups were studied: (1) unoperated and untreated rats served as controls, and (2) uninephrectomized rats received aldosterone (ALDO: 0.75 μg/h SC) through the implantation of a minipump plus 1% dietary NaCl and 0.2% KCl. Rats were anesthetized with pentobarbital (50 mg/kg) during uninephrectomy and minipump implantation. Rats were sacrificed at week 6 (n=7 in each group). The animal protocol was approved by University of Missouri-Columbia Animal Care and Use Committee. Our previous studies have shown no renal injury and fibrosis occurs in rats that receive only uninephrectomy or a high-salt diet. The normal rats, therefore, were used as controls in the study. Before they were killed, body weights were measured and systolic blood pressures were taken with a tail-cuff technique. Blood from the heart (3 mL) was collected into tubes containing 100 μL of 3.8% sodium citrate. Blood was centrifuged to separate plasma, and citrate was added for anticoagulation. After blood collection, plasma renin activity and creatinine studies. Kidneys were removed, weighted, frozen in isopentane with dry ice, and kept in a deep freezer for further studies.

Plasma Renin Activity
The measurement of plasma renin activity was performed with radioimmunoassay methods as previously described. The renin activity was determined through the estimation of the primary product of renin (Ang I) with a DuPont Ang IRIA kit. Plasma renin activity was expressed as nanograms of Ang I per milliliter plasma per hour of incubation.

Plasma Creatinine
Creatinine is generally regarded as the most useful endogenous substance to measure for the assessment of kidney function. Plasma creatinine concentration was detected with a biochemical assay as reported previously.

Renal Morphology
Cryostat sections (6 μm) were prepared to determine morphology with hematoxylin and eosin (H&E) staining, and fibrillar collagen accumulation was examined with collagen-specific picrosirius red (PSR) staining and observed with light microscopy as previously reported.

Immunohistochemical α-SMA Labeling
Cryostat sections (6 μm) were air dried, fixed in 10% buffered formalin for 5 minutes, and washed in PBS for 10 minutes. Then, they were incubated with primary anti-SMA antibody (Sigma Chemical Co) with a 1:400 dilution in PBS containing 1% BSA for 30 minutes and washed in PBS for 10 minutes. Sections were then incubated with IgG peroxidase–conjugated secondary antibody (Sigma Chemical Co) with a 1:150 dilution, washed in PBS for 10 minutes, incubated with 0.5 mg/mL diaminobenzidine tetrahydrochloride 2-hydrate plus 0.05% H2O2 for 10 minutes, and again washed in PBS. Negative control sections were incubated with secondary antibody alone. All sections were then stained with hematoxylin, dehydrated, mounted, and viewed with light microscopy.

 Autoradiographic Ang II Receptor Binding
Cryostat sections (16 μm) were preincubated for 15 minutes in sodium phosphate buffer and then incubated for 1 hour at room temperature in a fresh volume of the same buffer containing 0.2 μCi/mL (≈90 pmol/L) [125I]-[Sar9, Ile8]Ang II (Amersham), 2 g/L albumin, 0.4 mmol/L bacitracin, and 5 mmol/L Na2EDTA. Nonspecific binding was measured in the presence of 1 μmol/L unlabeled Ang II. To characterize Ang II receptor subtypes, sections were incubated with [125I]-[Sar9, Ile8]Ang II in presence of 10 μmol/L concentration of either the AT1 receptor antagonist losartan or the AT2 receptor antagonist PD123177. AT1 receptor binding was determined as that persisting in the presence of an excess of PD123177, whereas AT2 receptor binding was defined as that persisting in the presence of an excess of losartan. After incubation, sections were washed, dried, and exposed to Kodak NMB-6 film for 2 weeks.

 Autoradiographic ACE Binding
The radioligand used to label ACE was [125I]351A, a tyrosyl derivative of lisinopril and potent competitive inhibitor of ACE. 351A was iodinated according to the chloramine-T method and separated from free [125I] through SP Sephadex C25 column chromatography. Cryostat sections (16 μm) were incubated in 10 mmol/L sodium phosphate buffer, pH 7.4, containing 150 mmol/L NaCl and 2 g/L BSA with 0.3 μCi/mL (~300 pmol/L) [125I]351A for 1 hour at 20°C. Nonspecific binding was determined in parallel incubations containing 10 μmol/L unlabeled lisinopril. After incubation, sections were transferred through 4 successive 1-minute washes at 0°C, dried under a stream of cold air, placed in x-ray cassettes, and exposed to Kodak NMB-6 film for 3 days.

Quantification of Ang II Receptor and ACE Binding Density
The [125I] radioactivity standards (Amersham) were exposed to the same film in parallel with the tissue sections. Quantification of binding density was performed with a computer image analysis system (NIH Image 1.60). Radioactivity standards were corrected for decay and fitted to calibration curves through the computer to convert optical density values of each pixel into 125I radioactivity (expressed as dpm/mm²).

In Situ Hybridization
Cryostat sections (16 μm) were fixed in 4% formaldehyde for 10 minutes, washed with phosphate buffer (pH 7.4), and incubated in 0.25% acetic anhydride in 0.1 mol/L TE-HCl for 10 minutes. Sections were then hybridized (overnight at 45°C) with a random primed 35S-dATP-labeled type I collagen cDNA probe (1600-bp Psr1 insert of α1RI) (American Type Culture Collection) or 35S-labeled TGF-β1 cDNA (985-bp HindIII plus Shal; American Type Culture Collection). Sections were washed, dried, and subsequently exposed to Kodak Biomax x-ray film for 24 hours. After exposure, film was developed, and sections were stained with H&E. Quantification of mRNA optical density was performed with a computer image analysis system (NIH Image 1.60).

Distribution of ACE, Ang II Receptors, TGF-β1, and Type I Collagen mRNAs in the Kidney
After exposure, tissue sections were stained with H&E. The H&E-stained sections and α-SMA–labeled adjacent sections were over-
lapped with relevant film images and examined under light microscopy to determine the distribution of ACE, Ang II receptors, TGF-\(\beta\), and type I collagen mRNAs.

**Statistical Analysis**

Statistical analysis was performed with Student’s t test. Values are expressed as mean±SEM, with \(P<0.01\) considered statistically significant.

**Results**

**Body and Kidney Weights, Systolic Blood Pressure, and Plasma Renin Activity**

In ALDO-infused rats, body weights were significantly \((P<0.01)\) lower than those of the control rats. Systolic blood pressure, kidney weights, and plasma creatinine concentration were significantly \((P<0.01)\) increased, whereas plasma renin activity was markedly reduced \((P<0.01)\) in ALDO-infused rats (Table).

**Renal Morphology**

Compared with control rats (Figure 1A, H&E), a 6-week administration of ALDO in uninephrectomized rats on a high-salt diet was associated with diffuse renal injury involving both cortex and medulla. Bowman’s capsules of glomeruli and tubules were swollen, and glomerulosclerosis (not shown) and interstitial fibrosis was evident (Figure 1B, H&E). Collagen-specific PSR staining identifies interstitial fibrosis and collagen accumulation around the glomeruli (Figure 1D, PSR) compared with control kidneys, where only a small amount of fibrillar collagen is present in the tubulointerstitial space of both cortex and medulla (Figure 1C, PSR). Multiple sites of fibrosis are scattered in the kidney of ALDO-treated animals, but the extent of fibrosis at these sites varied.

Immunohistochemical analysis reveals that in normal kidneys, \(\alpha\)-SMA–positive cells are confined to the smooth muscle cells of arteries and arterioles, whereas cells of
glomeruli, tubules, and interstitial space are α-SMA negative (not shown). In ALDO-treated animals, abundant α-SMA–positive cells, or myofibroblasts (myoFb), were seen surrounding glomeruli and in the interstitial space of both cortex (Figures 1E and 1G) and medulla (Figure 1F). Negative control of α-SMA labeling is shown in Figure 1H. The density of α-SMA labeling varied within different sites of kidney, corresponding with the extent of tissue fibrosis.

**Ang II Receptor Binding**

In normal rat kidney, moderate-density Ang II receptor binding was observed in glomeruli and medulla, whereas low-density binding was seen in tubules and interstitial space in both the inner and outer cortex (Figure 2A). Renal Ang II receptor binding was totally displaced with losartan (Figure 2B) but was not affected by PD123177 (Figure 2C), indicating that the Ang II receptor subtype in normal rat kidney is predominantly of the AT1 subtype.

Compared with control rats, Ang II receptor binding in kidneys of rats receiving ALDO is significantly increased (P<0.01) at cortical and medullary sites of fibrosis (Figure 2D). In keeping with the variable extent of fibrosis in different sites of the kidney, Ang II receptor binding density was coincident with this heterogeneous distribution. High-density Ang II receptor binding was seen at sites of fibrous tissue. Ang II receptor binding in the repairing kidney was partially displaced with either losartan (60±4.5%) (Figure 1E) or PD123177 (40±4.2%) (Figure 1F), indicating the presence of both AT1 and AT2 receptor subtypes in sclerotic kidneys seen in association with ALDO administration.

**ACE Binding**

Moderate-density ACE binding was observed in the deep cortex of normal kidney, whereas very low-density ACE binding was present in the outer cortex and medulla (Figure 3A). In the kidneys of ALDO-treated animals, ACE binding density was markedly increased (P<0.01) at sites of cortical and medullary fibrosis (Figure 3B). ACE binding density was coincident with the extent of tissue fibrosis.

**TGF-β1 and Type I Collagen mRNA Expression**

Through in situ hybridization, we determined the localization and optical densities of TGF-β1 and type I collagen mRNA in the rat kidney and their spatial relationship to ACE and Ang II receptors. Very low, but uniform, expression of TGF-β1 mRNA was found throughout the cortex and medulla of normal rat kidneys (Figure 3C). In rats receiving ALDO, TGF-β1 mRNA expression was significantly (P<0.01) increased at sites of fibrosis (Figure 3D). TGF-β1 mRNA is accompanied by myoFb and macrophages.

The optical density of type I collagen mRNA was heterogeneous in the normal rat kidney: low in the cortex, moderate in the medulla, and high in the blood vessels and renal capsule (Figure 3E). In the kidney of ALDO-treated rats, type I collagen mRNA was markedly (P<0.01) elevated at cortical and medullary sites and colocalized with ACE and Ang II receptors.

Quantitative data for in vitro autoradiographic Ang II receptor and ACE binding density in the rat kidney of both experimental groups are shown in Figures 4A and 4B,
respectively. Quantitative data for TGF-β1 and type I collagen mRNAs in the rat kidney of both experimental groups are shown in Figures 5A and 5B, respectively.

**Discussion**

Long-term (≥4 weeks) inappropriate (relative to dietary salt and intravascular volume) elevations of circulating ALDO, together with an imposed high-salt diet in uninephrectomized rats, lead to reduced body weight and plasma renin activity, hypertension, and the appearance of myocardial fibrosis involving both the right and left atria and ventricles. Renal hypertrophy and damage followed by fibrosis are also evident as demonstrated and reported previously in this model.20 Even though the salt diet may increase the body water store, these rats had reduced food intake, which leads to decreased body weight. Increased plasma creatinine levels in ALDO-infused rats indicates renal dysfunction. The results of the present study have further confirmed the appearance of extensive renal fibrosis represented as glomerulosclerosis and interstitial fibrosis in ALDO-infused rats. However, the mechanisms responsible for such morphological changes in the kidney are uncertain.

The myoFb, which are also called repair tissue fibroblasts, play a central role in fibrous tissue formation in a diverse number of injured organs, including kidneys. For example, these cells contribute to collagen generation in renal fibrosis induced by unilateral ureteral obstruction and experimental glomerulonephritis.21,22 In the present study, abundant myoFb appeared in the interstitial space of renal cortex and medulla in rats treated with ALDO and colocalized with high density of type I collagen mRNA and accumulated collagen, indicating their role in collagen production in the repairing kidney. The myoFb aggregate at sites of fibrosis, which may be of benefit to fibrous tissue contraction.

Experimental and clinical evidence has implicated an important role for the circulating hormones, such as Ang II and ALDO of the RAAS, in the progressive destruction of nephrons in chronic cardiac failure and a wide variety of renal diseases. Clinical studies have noted that the long-term administration of an ACE inhibitor attenuates the progression
of renal failure in type I and II diabetics, as well as in patients with a primary glomerulopathy or adult polycystic kidney disease. Current evidence suggests that the beneficial effect of ACE inhibitors in renal diseases is primarily due to the inhibition of Ang II production. The present study has indicated that ACE and Ang II receptor binding densities were markedly elevated at sites of renal fibrosis in this rat model of hyperaldosteronism, which implicates activated local Ang II generation and the role of Ang II in fibrous tissue formation in the repairing kidney.

We previously reported in rats treated with ALDO that plasma renin was suppressed, whereas cardiac ACE binding was increased within fibrous tissue, as were renal ACE and Ang II receptor expressions observed in the present study. In the infarcted rat heart, ACE, Ang II content, and Ang II receptor binding densities were markedly increased at sites of fibrosis, whereas plasma renin and ACE remained unchanged. Hence, tissue ACE activity and Ang II receptor binding sites are independent of plasma renin, ACE, and Ang II.

In the repairing kidney, myoFb are anatomically coincident with the high binding density of ACE and Ang II receptors. These observations suggest that Ang II may regulate myoFb growth or their functions, such as collagen synthesis, in an autocrine/paracrine fashion. The present study also indicates that unlike in normal rat kidney, where the Ang II receptors are predominantly of AT₁ subtype, in the repairing kidney, ≈60% of Ang II receptors are AT₁, and the remainder are AT₂. AT₁ receptors have been considered responsible for the stimulation of cell growth and fibrous tissue formation. The functions of AT₂ receptors remain uncertain. It has been reported that AT₂ receptors may mediate the actions of anti-tissue growth through a reduction in DNA synthesis and cell proliferation. Mechanisms for the increase in AT₂ receptors in the repairing kidney are uncertain.

There is considerable evidence that Ang II promotes fibrogenesis. The effect of Ang II on fibrous tissue formation appears via its stimulation of TGF-β1 synthesis. Ang II increases TGF-β1 synthesis in cultured endothelial cells and myoFb. In vivo studies have further indicated that long-term losartan treatment in rats with myocardial infarction significantly attenuates mRNA expression of TGF-β1 and type I collagen at sites of cardiac fibrosis. In the fibrosed rat kidney induced by unilateral ureteral obstruction, TGF-β1 mRNA expression is largely increased, which could be abolished through pretreatment with the AT₁ receptor antagonist losartan. Moreover, the AT₁ receptor antagonist TCV116 reduced TGF-β1 mRNA levels, as well as those of collagens I and III and fibronectin, in hypertension-induced renal injury without a reduction in blood pressure. There have been numerous investigations into the role of TGF-β1 in tissue fibrosis; this fibrogenic cytokine has been implicated in the pathogenesis of fibrosis of kidney, liver, lung, skin, artery,
and heart in both animal and human models, where macrophages and fibroblasts/myofibroblasts are responsible for TGF-β1 production. Quantitative in situ hybridization provides the opportunity to investigate both mRNA level and its localization in tissue sections. Using the technique, we observed markedly increased TGF-β1 mRNA expression at sites of renal fibrosis in both the cortex and medulla of ALDO-infused rats. TGF-β1 mRNA expression at sites of repair is accompanied by macrophages and myofibroblasts, indicating their role in TGF-β1 synthesis. The high density of ACE and Ang II receptors is coexpressed with TGF-β1 mRNA. These findings indicate that anatomic requisites for locally generated Ang II and receptor binding could regulate the expression of TGF-β1 in an autocrine/paracrine manner.

In summary, with a model that simulates hyperaldosteronism, renal damage followed by fibrosis appears spatially coincident with markedly increased ACE and Ang II receptor binding densities at the sites of fibrosis and further colocalizes with TGF-β1 and type I collagen mRNA expression. The myofibroblasts are abundant at these sites of renal fibrosis and are accompanied by increased type I collagen and TGF-β1 mRNA expression and ACE and Ang II receptor binding. These observations suggest that local Ang II generation is likely activated and that Ang II may have a paracrine/autocrine function in the regulation of fibrous tissue formation in the remaining kidney of uninephrectomized rats receiving a high salt intake. The effect of Ang II on renal fibrogenesis may be accomplished through the regulation of TGF-β1 synthesis. The beneficial influence of ACE inhibitor or AT1 receptor antagonist in chronic heart failure may be attributed to the reduced expression of type I collagen and TGF-β1 and subsequent renal fibrosis and dysfunction.

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References

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