Angiotensin-Converting Enzyme Is Upregulated in the Proximal Tubules of Rats With Intense Proteinuria

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Abstract—Persistent proteinuria is considered a deleterious prognostic factor in most progressive renal diseases. However, the mechanisms by which proteinuria induces renal damage remain undetermined. Since proximal tubular cells possess all the machinery to generate angiotensin II (Ang II), we approached the hypothesis that proteinuria could elicit the renal activation of the renin-angiotensin system in a model of intense proteinuria and interstitial nephritis induced by protein overload. After uninephrectomy (UNX), Wistar-Kyoto rats received daily injections of 1 g BSA or saline for 8 days. The mean peak of proteinuria was observed at the fourth day (538±89 versus 3±1 mg/24 h in UNX controls; n=12; P<0.05) and was increased during the whole study period (at the eighth day: 438±49 mg/24 h; n=12; P=NS). Morphological examination of the kidneys at the end of the study showed marked tubular lesions (atrophy, vacuolization, dilation, and casts), interstitial infiltration of mononuclear cells, and mesangial expansion. In relation to UNX control rats, renal cortex of BSA-overloaded rats showed an increment in the gene expression of angiotensinogen (2.4-fold) and angiotensin-converting enzyme (ACE) (2.1-fold), as well as a diminution in renin gene expression. No changes were observed in angiotensin type 1 (AT₁) receptor mRNA expression in both groups of rats. By in situ reverse transcription–polymerase chain reaction and immunohistochemistry, ACE expression (gene and protein) was mainly localized in proximal and distal tubules and in the glomeruli. By immunohistochemistry, angiotensinogen was localized only in proximal tubules, and AT₁ receptor was localized mainly in proximal and distal tubules. In the tubular brush border, an increase in ACE activity was also seen (5.5±0.5 versus 3.1±0.7 U/mg protein ×10⁻⁴ in UNX control; n=7; P<0.05). Our results show that in the kidney of rats with intense proteinuria, ACE and angiotensinogen were upregulated, while gene expression of renin was inhibited and AT₁ was unmodified. On the whole, these data suggest an increase in Ang II intrarenal generation. Since Ang II can elicit renal cell growth and matrix production through the activation of AT₁ receptor, this peptide may be responsible for the tubulointerstitial lesions occurring in this model. These results suggest a novel mechanism by which proteinuria may participate in the progression of renal diseases. (Hypertension. 1999;33:732-739.)

Key Words: renin-angiotensin system ■ proteinuria ■ cells, tubular epithelial ■ interstitial damage

Progressive renal diseases are characterized by an interstitial infiltrate of mononuclear cells and the gradual destruction of the tubulointerstitial structure. It has been shown that there is a better correlation of renal function and disease progression with interstitial than with glomerular lesions.

The factors that cause the progression of intrinsic renal disease to end-stage renal failure are ill defined and poorly understood. However, persistent proteinuria is always considered an aggravating factor. In addition, in both human and experimental renal diseases, the degree of proteinuria is well correlated with the rate of progression of renal failure. Indeed, maneuvers that result in a reduction in the severity of proteinuria retard the development of progressive renal impairment.

The mechanisms by which proteinuria per se is associated with interstitial inflammation and fibrosis are unknown. Proximal tubular cells reabsorb proteins present in the tubular fluid and are thus vulnerable to the excessive and prolonged traffic of proteins. Recent evidence suggests that tubular protein overload upregulates and/or activates proinflammatory and vasoactive genes such as monocyte chemoattractant protein-1 (MCP-1), RANTES (regulated on activation, normal T cell expressed and secreted), and endothelin-1 (ET-1).

The renin-angiotensin system (RAS) has been implicated in the progression of renal damage. Angiotensin-converting enzyme (ACE) inhibition reduces proteinuria and limits progressive deterioration of renal function in a great variety
of renal diseases, independently of the presence of hypertension.1,4,5 However, the role of the RAS in the pathogenesis of tubulointerstitial lesion is not completely understood.

Since proximal tubular cells possess all the machinery to generate angiotensin II (Ang II),6 the main objective of this study was to establish whether proteinuria could activate some of the components of the RAS (ACE, angiotensinogen, renin, and angiotensin type 1 [AT1] receptor) in the kidney of rats with tubulointerstitial injury caused by protein overload. This nephritis is characterized by an early and impressive interstitial inflammation, and it has been proved that it is a valuable model to investigate the relationship between proteinuria and renal damage.7

Methods

Animal Model
Female Wistar-Kyoto rats (100 to 150 g) were fed standard rat chow ad libitum and given free access to water. Uninephrectomized (UNX) rats received daily injections of 1 g of BSA (Sigma) or saline for 8 days, as described.6 Periodically, 24-hour urine was collected for protein measurement by the sulfosalicylic acid method. At the end of the study, animals were anesthetized with pentobarbital sodium (5 mg/100 g body wt). Blood was collected, and kidneys were perfused with cold sodium saline and removed.

Renal Histopathological Studies
For light microscopy, paraffin-embedded sections (4 µm thick) were prepared and stained with hematoxylin-eosin and Masson's trichrome. For each animal, renal damage was graded from 0 to 4 by a semiquantitative score previously reported.8 All these studies were performed by 2 observers in a blinded fashion.

RNA Extraction and Reverse Transcription and Polymerase Chain Reaction
Pieces of renal cortex were homogenized, and total RNA was obtained by the acid guanidinium-phenol-chloroform method.9 Isolated RNA was reverse transcribed and then amplified with a commercial kit (Access RT-PCR System, Promega), with the use of specific primers for rat angiotensinogen (sense: 5'-TTCAAGGCAAAGCCTCCC-3'; antisense: 5'-CCAGCCGGG-GTGGCACT-3'; fragment: 308 base pair length), renin (sense: 5'-CGGTTGTTCTCACCACAAT-3'; antisense: 5'-GCCCATGCG-CAGACCCC-3'; fragment: 368 base pair length), ACE (sense: 5'-CCCTGATCAACCCAGGAGTTGGCAAGAG-3'; antisense: 5'-GCCAGCCTTCGlCCAGAAACGAC-3'; fragment: 317 base pair length), and AT1 (sense: 5'-TGGAACACGCTTGTTGGTAGT-3'; antisense: 5'-GCACAATGCCATCATTACCTC-3'; fragment: 607 base pair length) (a sequence with no divergence between AT1a and AT1b receptors) that were designed according to the published sequences.10-13 We performed RT-PCR of GAPDH as an internal standard.14 The optimum number of amplification cycles used for semiquantitative RT-PCR (30, 35, 33, and 22 cycles, respectively) was chosen on the basis of pilot experiments (data not shown). Then aliquots of each reaction were run on 4% acrylamide-bisacrylamide gels. The gels were dried and exposed to X-OMAT AS films (Eastman Kodak Company). Autoradiograms were quantified by scanning densitometry (Molecular Dynamics).

ACE, Angiotensinogen, and AT1 Receptor Immunohistochemical Studies
All immunohistochemical studies were performed by the avidin-biotin complex method, as described previously.5,15 Immunolocalization of ACE was performed with a monoclonal mouse anti-ACE antibody (Chemicon; 10 µg/mL), angiotensinogen with a polyclonal rabbit anti-rat angiotensinogen antibody (kindly provided by Dr C. Serria; 500-fold dilution), and AT1, receptor with a monoclonal mouse anti-rat AT1 receptor (kindly provided by Dr G.P. Vinson; undiluted). Control slides were treated with the corresponding nonimmune serum. Biotinylated rabbit anti-mouse IgG or biotinylated goat anti-rabbit IgG (Dako A/S) was used as a secondary antibody. The sites of phosphatase activity were visualized with fast red (for ACE staining; Dako A/S), and the sites of peroxidase activity were visualized with 3,3'-diaminobenzidine (for angiotensinogen and AT1 receptor staining; Sigma). Paraffin-embedded tissue sections were counterstained with Mayer's hematoxylin (Sigma).

In Situ RT-PCR
The cDNA was generated and then amplified with the use of the commercial kit Access RT-PCR System (Promega). In situ RT-PCR was performed essentially as described by Nuovo et al15 with minor modifications. Briefly, cryostat kidney sections of 5 µm thickness were air dried, fixed in acetone, and washed in Tris-buffered saline. Digoxigenin-labeled 11-dUTP (dig-dUTP, Boehringer Mannheim) was directly incorporated into the PCR products by addition of 10 µmol/L dig-dUTP to the RT-PCR mixture. The optimum number of amplification cycles used was chosen on the basis of pilot experiments that revealed at what time hybridization signal began to appear within nuclei (data not shown). Colorimetric detection of PCR products was performed in which the sections were incubated with an anti-digoxigenin antibody as described.8

The negative controls included samples pretreated with RNase A (150 µg/mL in 2× SSC) for 20 minutes at 37°C before in situ RT-PCR process or in situ RT-PCR done without primers.

Measurement of ACE Activity
Brush-border membranes were isolated from renal cortex, and ACE activity was determined in renal brush border and in serum by a spectrophotometric method (Sigma), as previously described.5

Statistical Analysis
Results are expressed as mean±SEM. Comparisons between 2 groups were made with the unpaired Student’s t test or the Kruskal-Wallis nonparametric ANOVA test when appropriate. Differences were considered significant if the P value was <0.05.

Results

Proteinuria
BSA-overloaded rats developed heavy proteinuria within the first 24 hours (112±37 versus UNX control rats, 2±1 mg/24 h; n=12; P<0.05), peaking on the fourth day (538±89 versus 3±1 mg/24 h; n=12; P<0.05). Proteinuria did not decrease significantly throughout the rest of the study period (at death: 438±49; n=12; P=N.S).

Morphological Lesions
At death, BSA-overloaded animals showed dramatic morphological kidney lesions, with marked interstitial infiltration and tubular atrophy and/or vacuolization, and protein casts within proximal and distal tubules (Figure 1). Only occasional necrotizing alterations, such as tubular brush-border loss or basement membrane detachment, were observed, as previously shown.16 BSA-overloaded rats also showed glomerular lesions consisting of mesangial hypercellularity and matrix expansion (Figure 1). The semiquantification of morphological lesions showed a significant increase in renal injury in BSA-overloaded rats.
with respect to UNX control animals (UNX control rats, 0.3±0.1; BSA-overloaded rats, 2.3±0.3; n=12; P<0.05).

Expression of RAS Genes in Renal Cortex
As shown in Figure 2 (top panel), a single band of the corresponding predicted size for each gene was obtained after RT-PCR on mRNA from renal cortex. Densitometric analysis of the bands showed an increase in ACE and angiotensinogen mRNA levels in BSA-overloaded rats compared with UNX control rats (Figure 2A and B). However, the renin gene expression was decreased in BSA-overloaded rats compared with UNX controls (Figure 2C). AT1 receptor mRNA expression was unchanged in the renal cortex after BSA overload (Figure 2D).

Renal Distribution of ACE, Angiotensinogen, and AT1 Receptor Immunoreactivity
In UNX control animals, ACE was mainly localized in proximal tubules, although it was also detected in glomeruli and in some distal tubules (Figure 3A and 3C). BSA-overloaded rats showed a significant increase in the ACE immunostaining, mainly in the proximal tubular cells and glomeruli (Figure 3B and 3D). Neither UNX control nor BSA-overloaded rats showed appreciable immunostaining of ACE in the renal medulla (data not shown).

By contrast, angiotensinogen was only localized in the proximal tubules in both UNX control and BSA-overloaded animals. However, the intensity of the staining was much more intense in the latter animals (Figure 4A and 4B).

In both groups of rats (UNX control and BSA-overloaded), AT1 receptor immunostaining was observed mainly in proximal and distal tubules (Figure 4C and 4D). In addition, cortical and medullary collecting ducts exhibited specific immunoreactivity. Only a weak immunostaining was observed in glomeruli and renal vasculature, probably because of the tissue fixation method.17

Localization of ACE mRNA
Since ACE is the key enzyme in the control of RAS, further studies were performed to localize its mRNA expression. In situ RT-PCR demonstrated that the localization of ACE mRNA staining was restricted to occasional tubular epithelial cells in UNX control rats (Figure 5A and 5C). In BSA-overloaded rats, there was an increase of the staining signal, especially in the proximal and distal tubules and in glomerular cells (Figure 5B and 5D). In the majority of the tubular epithelial cells, the perinuclear distribution of ACE mRNA can be appreciated in both groups of rats (Figure 5A and 5C). By contrast, no signal was detected in the inner medulla (not shown). The absence of detectable hybridization signal in the kidney sections where in situ RT-PCR was done without primers, as well as the decreased signal in the sections treated with RNase, confirmed that mRNA had been reverse transcribed and amplified during the in situ RT-PCR process (not shown).

ACE Activity in Kidney and Serum
To assess whether the increased ACE gene expression and protein synthesis corresponded with an increment in the activity of the enzyme, we determined ACE activity in both cortical brush-border membranes and serum. ACE activity in renal brush border of protein-overloaded rats was increased compared with UNX control and normal healthy rats (normal healthy rats, 2.8±0.7; UNX control rats, 3.1±0.7; BSA-overloaded rats, 5.5±0.5 U/mg protein ×10⁻⁴; n=7; P<0.05). By contrast, serum ACE activity was in the same range in all groups studied (normal healthy rats, 0.15±0.01; UNX control rats, 0.14±0.01; BSA-overloaded rats, 0.11±0.01 U/mL; n=7 per group; P=NS).

Discussion
In the present study we demonstrate that ACE is activated in the kidney of rats with persistent proteinuria. By in situ RT-PCR and immunohistochemistry, increased ACE (both
mRNA expression and protein) was mainly localized in proximal and distal tubules of renal cortex and in glomeruli. At the same time, the gene expression of angiotensinogen was increased, that of renin was inhibited, and that of AT1 was unmodified. By immunohistochemistry, angiotensinogen was only located in the proximal tubules, while AT1 was mainly localized in proximal and distal tubules.

Although we have not directly measured the Ang II levels, our data are not in contrast with an enhanced local Ang II production in the kidney of BSA-overloaded rats. In fact, in 2-kidney, 1 clip hypertensive rats and Ang II–infused rats, which show renal renin depletion, renal Ang II was higher than that found in normal kidneys, probably because of systemically delivered Ang I, which led to intrarenally formed Ang II, and because of the existence of renin-independent mechanisms in the kidney. The angiotensinogen upregulation in the kidney of BSA-overloaded rats could be due to a direct effect of proteinuria or to the action of Ang II. In cultured renal fibroblasts, we have reported that angiotensinogen gene expression was upregulated in response to Ang II. A similar phenomenon has been observed in hepatocytes and cardiac myocytes. Unexpectedly, we did not detect alterations in the AT1 receptor mRNA in the renal cortex after BSA overload, although it is possible that changes in the Ang II binding to the AT1 receptor could occur. Furthermore, Ang II downregulates AT1 receptor in

Figure 2. Top, mRNA expression of ACE, angiotensinogen (Ao), renin (REN), and AT1 receptor in renal cortex of UNX control rats (lanes 1 and 2) and BSA-overloaded rats (lanes 3 and 4). Two representative animals of each group are shown. Bottom, Densitometric analysis of renal cortex mRNA expression of ACE (A), angiotensinogen (B), renin (C), and AT1 receptor (D) in UNX control and BSA-overloaded rats. Each data point represents mean ± SEM; n = 12 animals per group. *P < 0.05 compared with UNX control rats.
rat vasculature and in cultured rat mesangial cells, while it upregulates this receptor in rabbit proximal tubules. In addition, we cannot rule out that cytokines present during renal inflammation may have a certain role. In fact, interleukin-1α caused an upregulation in AT1 receptors in rat vascular smooth muscle cells.

The incubation of cultured proximal tubular cells with different proteins, in concentrations found in the urine of patients with nephrotic syndrome, caused an upregulation in the synthesis of chemoattractant and vasoactive peptides (MCP-1, RANTES, and ET-1). In several experimental models of renal damage, characterized by heavy and sustained proteinuria, an increase in the expression of some proinflammatory genes was demonstrated, suggesting that proteinuria could be involved in this process. The mechanisms by which proteinuria induces the expression of a number of genes are unknown. However, the participation of the nuclear factor-κB (NF-κB) has been suggested. In vitro data show that NF-κB could be induced by the stress caused by protein accumulation in the endoplasmic cell reticulum. In this sense, data from our laboratory have shown that protein-overloaded rats had an increased NF-κB activity in the renal cortex (R. Largo et al, unpublished data, 1998). In conjunction with these data, an increased expression of MCP-1 and other inflammatory genes regulated under the control of NF-κB was noted in the renal cortex of those rats. In addition, a role for NF-κB in the gene regulation of angiotensinogen has been demonstrated.

The presence of important tubulointerstitial lesions associated with maintained proteinuria suggests that mediators generated in proximal tubular cells could be involved in this process. Locally generated Ang II could be secreted to the
interstitial space, inducing the vasoconstriction of peritubular vessels with subsequent ischemia. In addition, Ang II could activate renal interstitial fibroblasts, inducing several growth-related metabolic events mediated by the AT₁ receptor. Moreover, in vascular smooth muscle cells and mesangial cells, Ang II caused the activation of NF-κB and the production of MCP-1 and other chemokines involved in interstitial mononuclear cell recruitment. BSA-overloaded rats have an accumulation of matrix proteins in the interstitium, as well as increased synthesis of transforming growth factor-β (TGF-β) in the interstitial and cortical tubular cells. Since Ang II may participate in tissue fibrosis through TGF-β generation, our data suggest that this vasoactive peptide could be responsible, at least in part, for the tubulointerstitial lesions occurring in those animals. It is also possible that cytokines and growth factors (TGF-β, platelet-derived growth factor, ET-1, interleukin-6) released by Ang II could also have a certain role. In experimental models of renal injury, the treatment with ACE inhibitors and/or AT₁ receptor antagonist diminished renal TGF-β, platelet-derived growth factor, and ET-1 expression, coinciding with an improvement in renal lesions.

In summary, our results show that rats with intense proteinuria have an upregulation of ACE, the key enzyme in the control of the RAS, as well as an upregulation of angiotensinogen. The fact that both proteins were mainly located in the proximal renal tubules suggests that proteinuria may induce the local generation of Ang II and therefore may be responsible for the tubulointerstitial lesions observed in renal diseases associated with persistent proteinuria. On the whole, our data suggest a novel mechanism by which proteinuria may participate in the progression of renal diseases.
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