Activation of NF-κB in Tubular Epithelial Cells of Rats With Intense Proteinuria
Role of Angiotensin II and Endothelin-1

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Abstract—The mechanisms by which persistent proteinuria induces interstitial inflammation and fibrosis are not well known, although nuclear factor-κB (NF-κB), which regulates the transcription of many genes involved in renal injury, could be implicated. In rats with intense proteinuria, we studied the renal activation of NF-κB as well as the potential involvement of the vasoactive hormones angiotensin II (Ang II) and endothelin-1 (ET-1). Uninephrectomized Wistar-Kyoto rats receiving 1 g/d of BSA had proteinuria but no renal morphological lesions at day 1. By contrast, tubular atrophy and/or dilatation and mononuclear cell infiltration were observed after 8 or 28 days of BSA administration, coinciding with maximal proteinuria. In relation to control uninephrectomized rats, the renal cortex of nephritic rats showed an increment in the activation of NF-κB at all time periods studied. By in situ Southwestern histochemistry, NF-κB activity was mainly localized in proximal tubules, interstitial mononuclear cells, and, to a lesser extent, the glomeruli. The administration of the ACE inhibitor quinapril plus the ET A/ET B receptor antagonist bosentan during 28 days to BSA-overloaded animals diminished proteinuria, renal lesions, and NF-κB activity more markedly than single drugs. Cultured tubular epithelial cells exposed to BSA revealed an intense NF-κB activation in a time- and dose-dependent manner. Incubation of cells with receptor antagonists of Ang II (AT1: losartan and AT2: PD-123,319) or ET-1 (ET A: BQ123 and ET B: IRL1038) inhibited significantly the BSA-induced NF-κB activity (90%, 75%, 90%, and 60% of inhibition versus basal, respectively). Our results show that overload proteinuria causes NF-κB activation in tubular epithelial cells both in vivo and in vitro. The vasoactive peptides Ang II and ET-1 appear to be implicated in this effect. The results reveal a novel mechanism of perpetuation of renal damage induced by persistent proteinuria.


Key Words: proteinuria ■ epithelium ■ renal disease ■ angiotensin II ■ endothelin

Tubulointerstitial inflammation and the gradual deterioration of the renal function characterize progressive renal diseases.1 Several studies have demonstrated the importance of proinflammatory factors such as cytokines, adhesion molecules, growth factors, and vasoactive peptides in the progression of renal damage.1,2 Although the mechanisms leading to end-stage renal failure are not completely elucidated, persistent proteinuria is always considered an aggravating factor,1 suggesting that it could play a direct role in the development of renal injury.

The mechanisms by which proteinuria could cause interstitial inflammation and fibrosis are still not fully understood. In several experimental models of renal damage characterized by heavy and sustained proteinuria, the expression of vasoactive substances such as angiotensin II (Ang II) and endothelin-1 (ET-1) and chemokines such as monocyte chemottractant protein-1, RANTES, and osteopontin was demonstrated in renal tissue.1,3,4 The incubation of cultured proximal tubular cells with different proteins, in concentrations found in the urine of patients with nephrotic syndrome, led to the activation of proinflammatory molecules.5-7

In vitro studies have demonstrated that the expression of most of these factors is regulated by transcription factors, such as nuclear factor κB (NF-κB).6-9 In unstimulated cells, NF-κB is present as an inactive dimer bound to an inhibitory subunit (IκB). Different proinflammatory agents activate NF-κB by releasing IκB, resulting in the passage of NF-κB into the nucleus, where it binds to specific sequences in the promoter regions of target genes.10 NF-κB has been implicated in some inflammatory processes such as asthma and rheumatoid arthritis (reviewed in Reference 10). We have demonstrated that in a model of accelerated atherosclerosis in rabbits, there was an activation of NF-κB in the arterial wall, coinciding with an increase in the neointima formation and the infiltration of inflammatory cells.11 Proteinuria-induced chemokine production in cultured proximal tubular cells is mediated by NF-κB.6,7

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In this work, we studied whether proteinuria can directly activate NF-κB in a rat model of renal injury induced by protein overload and characterized by early and intense proteinuria associated to tubulointerstitial lesions. In addition, we localized the NF-κB activity in the rat kidney by in situ Southwestern histochemistry, a technique that allows detection of transcription factors in paraffin-embedded tissues, as recently described by our laboratory. Since we have reported that intense proteinuria can induce the production of Ang II and ET-1 in renal cortex of protein-overloaded rats, we approached the hypothesis that these vasoactive hormones could be involved in NF-κB activation induced by albumin.

Methods

Animal Model

Female Wistar 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. To assess the role of Ang II and ET-1 in the development of nephritis, we gave UNX animals daily injections of 1 or 0.5 g of BSA. After the first BSA injection, animals were treated with the ACE inhibitor quinapril (2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2'-bipyrimidin-4-yl]-benzene-sulfonamide, Hoffmann-La Roche Ltd). Quinapril (200 mg/L) was added to the drinking water and replaced every 48 hours, and bosentan (100 mg/kg in a dissolution of 5% arabic rubber) was given by gastric gavage once daily.

Periodically, all animals were housed in metabolic cages and 24-hour urine was collected for protein measurement. At the end of the studies, animals were anesthetized with sodium pentobarbital (5 mg/100 g body wt), 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. Glomerular (mesangial cell proliferation and matrix expansion) and tubulointerstitial injury (tubular dilation and/or atrophy, interstitial fibrosis, and inflammatory cell infiltrate) were graded by the following semiquantitative score: 0, no changes; 1, focal changes that involve 25% of the sample; 2, changes affecting >25% to 50%; 3, changes involving >50% to 75%; and 4, lesions affecting >75%. Two independent observers performed the semiquantification of morphological lesions in a blinded fashion.

Cell Cultures

The cell line NRK 52E, derived from rat kidney epithelial cells, was obtained from the American Type Culture Collection (Rockville, Md.). Cells were grown in DMEM (BioWhittaker) supplemented with 5% fetal calf serum (Gibco BRL), 60 μU/mL penicillin, 60 μg/mL streptomycin, and 2 mmol/L glutamine (BioWhittaker) at 37°C in the presence of 5% CO2. Cells were used between the 7th and 12th passages. In each experiment, cells were made quiescent for 48 hours in DMEM medium without fetal calf serum and stimulated at different times with BSA, Ang II (Sigma), ET-1 (Peninsula Laboratories), or phorbol 12-myristate 13-acetate (PMA, Sigma). In some experiments, cells were preincubated with lysine (Sigma) for 1 hour to inhibit the cellular protein uptake at the brush border membrane. In another group of experiments, cells were preincubated for 1 hour with two Ang II receptor antagonists: AT1, losartan (Dupont Merck) and AT2, PD-123,319 (RBI) (10^-6 mol/L); or with two ET receptor antagonists: ETa, BQ123 and ETb, IRL1628 (Neosystem) (10^-6 mol/L).

Extraction of Nuclear Proteins and Electrophoretic Mobility Shift Assay Studies

Nuclear extracts from renal cortex and cells were obtained as previously reported. The protein concentration of the extracts was quantified by the BCA method (Bio-Rad Laboratories). Electrophoretic mobility shift assays (EMSA) were performed with a commercial kit (Promega) as described. For supershift assays, 1 μg of anti-p50 or anti-p65 antibody (Santa Cruz Biotechnology) was added and incubated for 1 hour before the addition of the labeled probe.

Cellular Localization of NF-κB Activity

To localize the NF-κB activity in the rat kidney, in situ Southwestern histochemistry was performed on paraffin-embedded renal tissue sections from UNX control and BSA-overloaded animals, as previously described. For negative controls, samples were incubated with a mutant NF-κB probe or with a 100-fold excess of unlabeled consensus oligonucleotide. The number of cells positive to NF-κB activation in glomeruli (n=20 per biopsy) and in tubulointerstitium (per ×250 field, excluding glomeruli) was quantified with computer-assisted image analysis software (Optimas 6.5, Media Cybernetics) and digitized images.

Statistical Analysis

Results are expressed as mean±SEM. Comparisons between groups were made by unpaired Student’s t test or the Kruskal-Wallis nonparametric ANOVA test when appropriate. Differences were taken as significant at P<0.05.

Results

Development of Nephritis

The administration of 1 g BSA to UNX rats induced a marked increment in the urine protein excretion (Figure 1A). By light microscopy (semiquantitative analysis) no significant renal morphological lesions were seen at day 1. However, animals receiving BSA during 8 and 28 days developed marked tubular lesions (atrophy, vacuolization, dilatation, and casts), interstitial infiltration of mononuclear cells, and mesangial expansion (Figure 1, B through G).

Studies of NF-κB Activation in Renal Cortex

By EMSA studies, NF-κB activity was increased in the renal cortex of BSA-overloaded rats compared with that of UNX control animals at all time periods studied. NF-κB activity was increased at day 1 and remained high up to day 28 (Figure 2, lanes 1 to 6). The maximal NF-κB activation was found at day 8 (2.3-fold versus UNX controls). The presence of a 100-fold excess of unlabeled NF-κB oligonucleotide (Figure 2, lane 7), or the lack of nuclear proteins in the experiment (data not shown), inhibited the binding signal, confirming the specificity of the assay. At day 8, in nephritic rats the NF-κB complex consisted of dimers containing p50 and p65 subunits, because the preincubation for 1 hour with the respective antibodies reduced the intensity of the complex (Figure 2C). However, in rats with ureteral obstruction, NF-κB complexes containing subunit p65 were less prevalent than complexes containing p50, p52, c-Rel, or RelB, whereas in rats with immune complex nephritis, c-Rel was not present in the activated NF-κB. Although these data may suggest that the different composition of NF-κB dimers could be determinant in the expression of different genes, further studies are needed in this regard.
Cellular Localization of NF-κB Activation

In UNX control animals, NF-κB activity was localized in proximal tubules (8 days: 34±15 cells per field) and to a lesser extent in glomeruli (0.23±0.02 cells per glomerulus, n=7). The administration of BSA to UNX rats induced an increment in the number of NF-κB-positive cells both in tubules (24 hours: 135±30; 8 days: 194±33 cells per field; P<0.05 in respect to UNX control, n=7 per group) and glomeruli (24 hours: 37±18; 8 days: 51±14 cells per glomerulus; P<0.05 in respect to UNX control, n=7 per group) (Figure 3, A and B). At 8 days, in BSA-overloaded animals, renal interstitial cells (which were not detected either in UNX controls or BSA-overloaded rats at 24 hours) also showed NF-κB activation (93±23 cells per field, n=7) (Figure 3B). Neither the incubation of the sections with a mutant NF-κB probe (data not shown) nor with a 100-fold excess of unlabeled consensus oligonucleotide (Figure 3C) showed any positive staining, indicating the specificity of the technique.

Role of Ang II and ET-1 on Proteinuria, Morphological Lesions, and NF-κB Activation in Nephritic Rats

As mentioned above, rats receiving 1 g of BSA had severe proteinuria and marked tubulointerstitial lesions. In early experiments, these animals were treated with quinapril or bosentan, as indicated in the Methods section. However, no effect was noted either on proteinuria or on morphological lesions. When both drugs were given in combination, a certain improvement was noted, but only at day 8 (data not shown). Because of the severity of the lesions observed, we performed new experiments, injecting a half-dose of BSA (0.5 g/d). In these conditions, animals also showed proteinuria and renal lesions, although less severe than those
Effect of Albumin on NF-κB Activation in Cultured Tubular Epithelial Cells

As previously reported, albumin causes NF-κB activation in cultured tubular epithelial cells. In our experimental conditions, BSA overload (30 mg/mL) induced NF-κB activation in a time-dependent manner, being the maximal effect after 30 minutes of incubation (Figure 5A). NF-κB activation was already noted with 1 mg/mL BSA for 30 minutes (Figure 5B). The preincubation of the cells with 100 mmol/L lysine inhibited the BSA-induced NF-κB activity, indicating that this effect was specific for BSA (Figure 5B).

Role of Ang II and ET-1 on the NF-κB Activation in Tubular Epithelial Cells

In parallel experiments, we detected by reverse transcription–polymerase chain reaction that tubular epithelial cells NRK 52E have a basal mRNA expression of both Ang II receptors (AT₁ and AT₂) and ET-1 receptors (ET₆ and ET₈) (data not shown).

Cells were preincubated for 1 hour with losartan (AT₁ receptor antagonist), PD-123,319 (AT₂ receptor antagonist), BQ123 (ET₆ receptor antagonist), or IRL1038 (ET₈ receptor antagonist) before stimulation with 30 mg/mL BSA or 10⁻⁷ mol/L PMA for 30 minutes. The presence of the Ang II and ET-1 receptor antagonists in the incubation media significantly inhibited the NF-κB activity caused by BSA but not that induced by PMA (Figure 5C), indicating that this effect is rather specific and therefore not related to all NF-κB activators. Neither Ang II nor ET-1 receptor antagonists alone had any significant effect on NF-κB activation in our experimental conditions. In addition, the stimulation of tubular epithelial cells with Ang II (10⁻⁷ mol/L) and ET-1 (10⁻⁷ mol/L) increased NF-κB activity in a time-dependent manner, with the maximal NF-κB activity after 30 minutes of stimulation (Figure 6, A and C). The Ang II–induced NF-κB activation was inhibited with the preincubation of cells with losartan and PD-123,319 (Figure 6B). The presence in the culture medium of BQ123 and IRL1038 abrogated the ET₁–induced NF-κB activation (Figure 6D).

Discussion

Progressive renal failure is characterized by proteinuria, inflammation, and scarring in the interstitium. Much evidence supports a role for proteinuria per se in the development of interstitial fibrosis, albeit the molecular mechanisms are still poorly understood. We first investigated whether in vivo protein overload could directly cause an increment in the renal NF-κB activity. This nonimmunological nephritis is considered a valuable model to investigate the relation between proteinuria and renal damage. In relation to UNX controls, BSA-overloaded rats showed an increment in NF-κB activation compared with UNX control rats (Figure 4A). By light microscopy (semiquantitative analysis), untreated rats showed important renal lesions (interstitial infiltrate, tubular atrophy, protein casts within the proximal and distal tubules, and mesangial expansion and proliferation) that were significantly attenuated by quinapril/bosentan treatment (0.6 versus untreated rats: 1.9 P<0.01; n=7 per group) but not by quinapril or bosentan alone, although a certain improvement was noted (0.9±0.2 and 1.1±0.2, respectively; P=NS; n=7 per group) (Figure 4, B through E). As expected, 0.5 g BSA-overloaded rats had increased NF-κB activation compared with UNX control rats, which was significantly decreased in quinapril-treated and quinapril/bosentan-treated rats but not in bosentan-treated rats (Figure 4, F and G).
renal NF-κB activation was also observed in rats with remnant kidney or rats with immune complex glomerulonephritis.\textsuperscript{16,18} Therefore, it could be speculated that intense proteinuria increased NF-κB activity in tubular epithelial cells and upregulated many NF-κB–dependent inflammatory genes. In fact, increased expression of monocyte chemoattractant protein-1, RANTES, and adhesion molecules was noted in experimental proteinuric renal diseases (reviewed in Reference 1).

Because the mechanisms by which intense proteinuria could induce NF-κB activation and the expression of a number of genes are not yet well understood, we approached the hypothesis that the vasoactive peptides Ang II and ET-1...
could be implicated in this phenomenon. ACE inhibitors are considered the best therapy available to date for proteinuric progressive nephropathies.\(^1\) In this regard, ACE inhibitors prevent proteinuria and NF-\(\kappa\)B activation in some models of renal injury.\(^{15,16,18}\) In this report, we demonstrate that the administration of quinapril plus the ETA/ETB receptor antagonist bosentan during 28 days to UNX animals receiving 0.5 g BSA was associated with a diminution in proteinuria, renal lesions, and NF-\(\kappa\)B activation more markedly than each one individually. The mechanisms of the beneficial effect when both drugs were given in combination were not defined in this report. We can speculate that ACE inhibitors could limit, at least partially, glomerular permeability to proteins (as it is already known), whereas bosentan could prevent the effect of enhanced ET-1 synthesis on tubulointerstitial cells. In fact, the challenge of cultured tubular epithelial cells with different proteins upregulates ET-1 expression, which was mostly secreted toward interstitial compartment.\(^3,4\) ET-1 stimulates both interstitial fibroblast proliferation and extracellular matrix synthesis and has potent chemotactic properties on monocytes/macrophages.\(^1,19\) Thus, we have reported an increment in the production of ET-1 in proximal tubules of rats after 8 days of 1 g BSA administration,\(^3,4\) coinciding with the increased NF-\(\kappa\)B activity in the renal cortex. In addition, it has been demonstrated that the beneficial effects of ACE inhibitors in the pathogenesis of renal damage could be a

\[\text{Figure 4. Role of Ang II and ET-1 on proteinuria (A), morphological lesions (B through E), and NF-\(\kappa\)B activity (F, G) in 0.5 g BSA–overloaded rats at day 28. A, Proteinuria in nontreated BSA–overloaded rats (white column), } P<0.05 \text{ with respect to BSA–overloaded rats. B, Tubular atrophy and inflammation (inserted panel) shown in BSA–overloaded rats were only partially attenuated in quinapril (Quin)-treated (C) and bosentan (Bos)-treated rats (D). By contrast, rats treated with quinapril/bosentan did not show significant renal lesions (E). F, NF-\(\kappa\)B activity of UNX control (lanes 1, 2), BSA-overloaded (lanes 3, 4), quinapril-treated (lanes 5, 6), bosentan-treated (lanes 7, 8), and quinapril/bosentan-treated rats (lanes 9, 10). Autoradiography shows 2 representative animals from each group. G, Densitometric analysis of NF-\(\kappa\)B bands. Individual bar values are mean±SEM of each group, } n=7 \text{ animals per group. } *P<0.05 \text{ with respect to BSA–overloaded rats.}\]
result of decreased Ang II and ET-1 generation.20–22 In the same way, endothelin blockade prevents the cardiovascular and renal effects of Ang II.23 However, because proteinuria, renal lesions, and NF-κB activation were not fully prevented by the combination of quinapril and bosentan, other mechanisms may be implicated. In fact, numerous cytokines and chemokines are produced by tubular cells when they are overloaded with various proteins.1,5–7 In rats with severe proteinuria and renal damage induced by adriamycin, the administration of pyrrolidine dithiocarbamate totally inhibited the cortical NF-κB activation, although interstitial monocyte/macrophage infiltration and tubular injury were only partially reduced.17 In this sense, recent studies have shown that the simultaneous administration of the immunosuppressors mycophenolate mofetil with an ACE inhibitor or an AT 1 receptor antagonist to remnant kidney rats afforded better renal protection than each single drug.24,25

Preincubation of cultured tubular epithelial cells with Ang II and ET-1 specific receptor antagonists inhibited the BSA-induced NF-κB activity. In addition, in these cells, Ang II and ET-1 increased NF-κB activity through AT 1 receptor and AT 2 receptors and ET A and ET B receptors, respectively, as in the case of BSA-induced NF-κB activity. Although most of the known effects of Ang II appear to be mediated by the activation of the AT 1 receptors, recent evidence suggests a functional role for the AT 2 receptor in the kidney.2,26 In the rat model of ureteral obstruction, both AT 1 and AT 2 receptor antagonists decreased NF-κB activation in the obstructed kidney, though this effect was greater with the AT 1 antagonist.27 Furthermore, we have recently reported that the administration of the AT 1 antagonist losartan or the AT 2 antagonist PD123,319 to rats receiving an infusion of Ang II (subcutaneously by osmotic minipumps) inhibited the Ang II–induced NF-κB activation, but only losartan diminished AP-1 activation.28 In vitro, Ang II caused the activation of NF-κB through its AT 1 and AT 2 receptors in different cell types.29,30 We have recently reported that in vascular smooth muscle cells, both receptors share some signaling pathways (oxygen radicals and ceramide). However, tyrosine kinase only participates in NF-κB activity induced by AT 1 activation.29

On the whole, our results demonstrate that protein overload increases the activity of the transcription factor NF-κB in tubular epithelial cells both in vivo and in vitro. Together with previous findings, the vasoactive peptides Ang II and ET-1 appear to be implicated in the tubulointerstitial injury induced by proteinuria. The present results reveal a novel mechanism of perpetuation of renal damage induced by persistent proteinuria.

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