Targeted Deletion of Angiotensin II Type 1A Receptor Does not Protect Mice from Progressive Nephropathy of Overload Proteinuria

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Abstract. In experimental and human renal diseases, progression is limited by angiotensin-converting enzyme inhibitors. Whether renoprotection was due to their capacity of reducing proinflammatory and profibrotic effects of angiotensin II (Ang II) or limiting proteinuria and its long term toxicity is debated. For dissecting the relative contribution of Ang II and proteinuria to chronic renal damage, the protein-overload proteinuria model was used in genetically modified mice lacking the major isoform of murine AT1 receptor (AT1A). Uninephrectomized mice received a daily injection of BSA or saline for 4 or 11 wk. AT1A+/−−/BSA mice acquired a renal phenotype of proteinuria and renal glomerular and tubulointerstitial lesions, albeit attenuated with respect to AT1A+/−+BSA. Administration of the calcium channel blocker lacidipine to reduce BP of AT1A+/−+BSA mice to levels of AT1A−/−−BSA translated into comparable values of protein excretion rate and glomerular and tubulointerstitial injury in both strains. These results confirm that the toxic effect of protein trafficking on renal disease progression is not necessarily dependent on Ang II to the extent that targeted deletion of AT1A does not prevent disease progression. A role of Ang II via AT1B or AT2 receptors is still a possibility that cannot be ruled out by the present experimental approach. These findings provide a clear rationale for specifically targeting proteinuria in pharmacologic interventions of chronic nephropathies.

Chronic kidney diseases are emerging as a worldwide public health problem as a result of the increase at an alarming rate of the number of patients who are developing end-stage renal failure and the consequent financial burden for the health care budget that soon will become unaffordable even for industrialized countries (1–3).

Progression to renal parenchymal damage and ESRD, which seems to be largely independent of the initial insult, is the final common pathway for chronic proteinuric nephropathies in animals and humans (4). The key event is enhanced glomerular capillary pressure; this impairs glomerular permeability to proteins and permits excessive amounts of proteins to reach the lumen of the proximal tubule (4,5). The secondary process of tubular reabsorption of filtered proteins can contribute to renal interstitial injury by activating intracellular events, including upregulation of the genes encoding vasoactive and inflammatory mediators (5). Thus, proteinuria is a major determinant of disease progression in both experimental and human nephropathies to the extent that the degree of urinary protein excretion correlates with the magnitude of renal damage in experimental models, and proteinuria reduction helps to preserve renal function (4). Both interstitial inflammation and progression of renal disease can be controlled by drugs that inhibit the renin-angiotensin system (RAS), such as angiotensin-converting enzyme (ACE) inhibitors or angiotensin II (Ang II) type 1 receptor antagonists, which strengthen the glomerular permeability barrier to proteins and thereby limit proteinuria and filtered protein-dependent inflammatory signals.

Emerging evidence indicates that Ang II—the main effector of the RAS—also stimulates the synthesis of extracellular matrix proteins, inflammatory cytokines, and growth factors by renal tubule cells, eventually contributing to interstitial inflammation and scarring (6–8). Together, these findings have been echoed in the debate of whether renoprotection afforded by RAS blockers is due to their capacity to limit excess protein ultrafiltration and its deleterious consequences or through the alternative but not mutually exclusive pathway of reducing the proinflammatory and profibrotic effects of Ang II. So far, however, this question remains unanswered because abnormal glomerular protein trafficking and activation of renin-angiotensin cascade are so interrelated that dissecting their relative contribution to chronic renal damage has been difficult.

The biologic roles of Ang II are elicited through binding to
specific type 1 (AT$_1$) and type 2 (AT$_2$) receptors. Among the AT$_1$ receptors, two subtypes, AT$_{1A}$ and AT$_{1B}$, have been identified in human, rat, and mouse (9,10). These receptors are products of separate genes and share substantial sequence homology (11). In mice, the AT$_{1A}$ receptor is the major AT$_1$ identified in human, rat, and mouse (9,10). These receptors are

**Materials and Methods**

**Mice**

Mice lacking AT$_{1A}$ receptor for Ang II were produced using homologous recombination in embryonic stem cells as described previously (13). Animals were bred in the Animal Facility at the Durham Veterans Affairs Medical Center under National Institutes of Health guidelines. Genotypes were determined by Southern blot analysis of DNA obtained from tail biopsies as described (14). Mice used in these studies were F1 animals generated from intercrossing of AT$_{1A}$(+/-) C57BL/6 with AT$_{1A}$(+/-) 129/J animals. The AT$_{1A}$ mutation was back-crossed for >12 generations onto each of these backgrounds, which is more than sufficient to obtain a >99% inbred line. The F1 animals therefore are genetically identical (except for the AT$_{1A}$ gene locus) and are genetically homogeneous. AT$_{1A}$(+/-) have reduced BP compared with wild-type littermates; renal morphology is essentially preserved with the exception of marked hypertrophy of the juxtaglomerular apparatus and modest expansion of the glomerular mesangium (15). Animal care and treatment were conducted in accordance with the institutional guidelines that are in compliance with national (Decreto Legislativo n.116, Gazzetta Ufficiale suppl 40, 18 febbraio 1992, Circolare n.8, Gazzetta Ufficiale 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL358-1, December 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). Animals were housed at a constant room temperature with a 12-h dark/12-h light cycle and were fed a standard diet.

**Murine Model of Protein-Overload Proteinuria**

BSA-overload proteinuria was induced in AT$_{1A}$(+/-) and AT$_{1A}$(-/-) mice that were uninephrectomized under anesthesia 5 d before starting BSA injections (16). Low-endotoxin BSA (Sigma-A-9430) was dissolved in saline and given 5 d weekly intraperitoneally at the dose of 10 mg/g body wt for 4 or 11 wk. Unilateral nephrectomy per se did not affect proteinuria or glomerulosclerosis in AT$_{1A}$(+/-) and AT$_{1A}$(-/-) animals that were given saline (proteinuria, mg/d, AT$_{1A}$(+/-): 8 ± 0.8 versus 7 ± 1; AT$_{1A}$(-/-): 6 ± 1 versus 7 ± 0.4; glomerulosclerosis, %., AT$_{1A}$(+/-): 0 versus 0.2 ± 0.2 AT$_{1A}$(-/-): 0 versus 0), which is in line with previous observations that genetic background is an important factor determining susceptibility to the development of renal damage in mice (17,18). During the study, nonuninephrectomized mice were used as controls. AT$_{1A}$(+/-) uninephrectomized BSA mice were actual controls for AT$_{1A}$(-/-) uninephrectomized BSA ones.

**Experimental Design**

**Short-Term Experiments.** Male mice were divided as follows: AT$_{1A}$(+/-) (n = 11) and AT$_{1A}$(-/-) (n = 7) mice received daily intraperitoneal injections of BSA (10 mg/g) and were killed at 4 wk. AT$_{1A}$(+/-) (n = 6) and AT$_{1A}$(-/-) (n = 6) mice that received saline were followed for 4 wk and used as controls. Systolic BP (SBP), serum blood urea nitrogen (BUN) and urinary excretion of endothelin-1 (ET-1) were measured at 4 wk. Urinary protein excretion was monitored along the study. When the mice were killed, kidneys were removed and processed for histologic analysis and renal expression of ET-1 mRNA and protein.

**Long-Term Experiments.** AT$_{1A}$(+/-) (n = 11) and AT$_{1A}$(-/-) (n = 7) mice received daily intraperitoneal injections of BSA (10 mg/g) and were killed at 11 wk. For ruling out that possible difference in renal injury between BSA-treated AT$_{1A}$(+/-) and AT$_{1A}$(-/-) mice could be due to the different BP levels previously reported in the two strains (14), an additional group of AT$_{1A}$(+/-) (n = 7) mice were treated daily with the dihydropyridinic calcium channel blocker lacidipine (3 mg/kg) by gavage (19). AT$_{1A}$(+/-) (n = 6) and AT$_{1A}$(-/-) (n = 6) mice that received saline were followed for 11 wk and used as controls. SBP, urinary protein excretion, and serum BUN were assessed at 11 wk. When the mice were killed, kidneys were removed and processed for histologic analysis and evaluation of F4/80-positive monocyte/macrophage accumulation in the renal interstitium.
solution, and development with 3,3-diaminobenzidine tetrahydrochloride. The sections were counterstained with Harris hematoxylin. Negative controls were obtained by omitting the primary antibody. F4/80-labeled cells were counted in 30 randomly selected high-power microscopic fields (×400) per animal.

**Immunolocalization of ET-1**

Kidney sections that were fixed in Duboscq-Brazil were deparaffinized and dehydrated. Microwave heating of tissue sections was performed twice for 5 min in citrate buffer with an operating frequency of 2450 MHz and 600-W power output. After blocking for 30 min in 1% BSA (Sigma), the sections were incubated overnight with the rabbit anti-ET-1 primary antibody (Peninsula Laboratories, San Carlos, CA; 1:500) at 4°C. Then they were rinsed with PBS and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories; 1:200) for 30 min at room temperature. Alkaline phosphatase–conjugated streptavidin (Roche Diagnostic) was applied at a dilution of 1:50 for 30 min. The reaction product was visualized by incubation with Fast Red substrate (Vector Laboratories) as described (21). Counterstaining was performed by using Harris hematoxylin (Bio-Optica, Milan, Italy). Negative controls included the use of anti-ET-1 antibody absorbed for 4 h with synthetic ET-1 or nonimmune serum instead of the primary antibody. Signal intensity was graded on a scale of 0 to 3 (0, no staining; 1, weak; 2, moderate; 3, strong intensity).

**Real-Time PCR**

Total RNA was extracted from the whole-kidney tissue by the guanidium isothiocyanate/cesium chloride procedure. Contaminating genomic DNA was removed by RNase-free DNase (Promega, Madison, WI) and treated with RNase-free DNase (Promega, Madison, WI). Total RNA was reverse transcribed using random exmers oligonucleotides and 200 U of SuperScript II RT (Life Technologies, San Giuliano Milanese, Italy) for 1 h at 42°C. The purified RNA (1 μg) was reverse-transcribed using random exmers oligonucleotides and 200 U of SuperScript II RT (Life Technologies, San Giuliano Milanese, Italy) for 1 h at 42°C. No enzyme was added for reverse transcription–negative controls.

Real-time PCR was performed on Gene Amp ABI 5700 Sequence Detection System (PE Biosystems, Warrington, UK) using heat-activated TaqDNA polymerase (Amplitaq Gold; PE Biosystems). The SYBR Green I PCR Reagents kit was used according to the manufacturer’s protocol. After an initial hold of 2 min at 50°C and 10 min at 95°C, the samples were cycled 40 times at 95°C for 15 s and 60°C for 1 min to reach the plateau. Fluorescence detection, defined as threshold cycle (Ct), is automatically picked in the exponential phase of the PCR and used for the relative quantification of the target gene. The comparative Ct method normalizes the number of target gene copies to a housekeeping gene as glyceraldehyde-3-phosphate dehydrogenase (ΔCt). Gene expression was then evaluated by the quantification of cDNA corresponding with the target gene relative to a control sample serving as a physiological reference (e.g., AT1A+/+ or AT1A−/− given saline, ΔΔCt). On the basis of exponential amplification of the target gene, the amount of amplified molecules at the Ct is given by 2−ΔΔCt. The following oligonucleotide primers (300 nM) were used: mouse ET-1 sense 5′-AACTCGAAGGTTGGAGGC-3′ ( Biosystems), antisense 5′-CAGGAAAAAGTGCCTTTAGGC-3′; mouse glyceraldehyde-3-phosphate dehydrogenase sense 5′-CTCATCCTGTACCT-3′ (Biosystems), antisense 5′-CTGGGATGGCTTTGCCCAC-3′. All primers were obtained from Sigma Genosys (Cambridgeshire, UK). True identity of the amplification products was ensured by primer specificity for ET-1 rat sequence, the presence of a single dissociation curve at a constant T melting, and the lack of genomic DNA contamination or primer dimers in RT− samples.

**Urinary ET-1 Measurement**

Urinary ET-1 levels were evaluated by RIA after extraction with Sep-pak C18 (Waters, Milford, MA) as described previously (22). Cross-reactivity of the ET-1 antibody (Peninsula Laboratories, San Carlos, CA) was as follows: ET-2, 46.9%; ET-3, 17%; and big-ET-1, 9.4%. Results corrected for the recovery were expressed as pg/d.

**Statistical Analyses**

Data are expressed as mean ± SEM. Data were analyzed using the nonparametric Kruskal-Wallis test for multiple comparisons. The statistical significance level was defined as P < 0.05.

**Results**

**Short-Term Experiments**

**Proteinuria and Glomerulosclerosis in AT1A−/− Protein-Overload Mice.** To study the role of the AT1A receptor in renal damage associated with proteinuria, we used the model of protein-overload nephropathy. After unilateral nephrectomy to accelerate renal injury, wild-type AT1A+/+ and AT1A−/− knockout littermates received daily injections of BSA. Proteinuria increased within 2 d after BSA and remained sustained thereafter in both AT1A+/+ (AT1A+/+ + BSA) and AT1A−/− (AT1A−/− + BSA) mice. At the end of the 4-wk BSA treatment, protein excretion rate was significantly increased in both groups compared with AT1A+/+ and AT1A−/− mice that received saline (Figure 1A). Urinary protein excretion in AT1A+/+ + BSA was numerically higher but not significantly different than in AT1A−/− + BSA littersmates (Figure 1A). Morphologic evaluation of the kidneys of AT1A+/+ + BSA mice by light microscopy showed segmental areas of glomerulosclerosis affecting 8.3 ± 2% of glomeruli (Figure 1B), abundant protein casts, and tubular damage (score, 1 ± 0). In AT1A−/− + BSA mice, focal and segmental glomerulosclerosis involved only 3.2 ± 1% of glomeruli, and the extent of glomerular sclerosis was significantly lower than in AT1A−/− + BSA littersmates (Figure 1B). Tubular damage was variably present in AT1A+/+ + BSA and AT1A−/− + BSA mice. Glomerular and tubulointerstitial damage was absent in the AT1A+/+ and AT1A−/− mice that received saline.

**BP in AT1A−/− Protein-Overload Mice.** Type 1A Ang II receptor function is required for vascular and hemodynamic responses to Ang II (23), and altered expression of AT1A gene type and knockout mice. At the end of the follow-up period, BUN values were comparable in AT1A−/− mice and AT1A+/+ + BSA, but proteinsuric and AT1A−/− mice received daily injections of BSA. Proteinuria increased within 2 d after BSA and remained sustained thereafter in both AT1A+/+ (AT1A+/+ + BSA) and AT1A−/− (AT1A−/− + BSA) mice. At the end of the 4-wk BSA treatment, protein excretion rate was significantly increased in both groups compared with AT1A+/+ and AT1A−/− mice that received saline (Figure 1A). Urinary protein excretion in AT1A+/+ + BSA was numerically higher but not significantly different than in AT1A−/− + BSA littersmates (Figure 1A). Morphologic evaluation of the kidneys of AT1A+/+ + BSA mice by light microscopy showed segmental areas of glomerulosclerosis affecting 8.3 ± 2% of glomeruli (Figure 1B), abundant protein casts, and tubular damage (score, 1 ± 0). In AT1A−/− + BSA mice, focal and segmental glomerulosclerosis involved only 3.2 ± 1% of glomeruli, and the extent of glomerular sclerosis was significantly lower than in AT1A−/− + BSA littersmates (Figure 1B). Tubular damage was variably present in AT1A+/+ + BSA and AT1A−/− + BSA mice. Glomerular and tubulointerstitial damage was absent in the AT1A+/+ and AT1A−/− mice that received saline.
Renal ET-1 in AT1A−/− Protein-Overload Mice. In animal models of proteinuric progressive kidney disease, the renal synthesis of ET-1 is increased (24). Moreover, in double transgenic rats that harbor both human renin and angiotensinogen genes, ET-1 mediates inflammatory processes related to Ang II–induced heart and kidney tissue damage (25). We therefore determined the effect of protein overload on intrarenal ET-1 gene expression and synthesis in AT1A knockout mice. Four weeks of BSA injections stimulated transcription of ET-1 gene in kidneys of AT1A+/+ mice; ET-1 mRNA levels were threefold increased with respect to AT1A+/+ mice that received saline (Figure 3A). A similar upregulation of ET-1 gene expression was observed in AT1A−/− BSA animals (Figure 3A). These changes in intrarenal ET-1 gene expression were accompanied by enhanced synthesis of ET-1 peptide. In this regard, urinary ET-1 excretion—which reflects the renal synthesis of the peptide (22)—was significantly increased in AT1A+/+ BSA mice (Figure 3B). Similarly, ET-1 excretion rate was increased to a similar extent in protein-overloaded AT1A−/− BSA animals (Figure 3B). In both groups, the urinary ET-1 was significantly higher than in the corresponding AT1A+/+ and AT1A−/− animals that received saline. To assess the distribution of ET-1 protein within the kidney, we performed immunohistochemistry experiments, and representative pictures are shown in Figure 4. In AT1A+/+ and AT1A−/− animals that received saline, a weak staining was found in tubular epithelial cells and parietal epithelial cells. Tubular staining was more intense in some cortical areas. Results of semiquantitative assessment of ET-1 staining were comparable in these groups (1 ± 0.2 versus 1.2 ± 0.1). ET-1 staining markedly and significantly (P < 0.01) increased in the kidneys of protein-overloaded AT1A+/+ and AT1A−/− animals both in tubular cells and, to much lesser extent, in glomerular epithelial cells. Mononuclear cells in some focal infiltrates showed strong ET-1 staining. Results of semiquantitative analysis showed significant (P < 0.01) differences as compared with animals that were given saline (AT1A+/+ BSA, 1.8 ± 0.1; AT1A−/− BSA, 2.6 ± 0.2).

Long-Term Experiments

Effect of Comparable BP on Renal Damage in AT1A−/− and AT1A+/+ Protein-Overload Mice. Mice with targeted disruption of AT1A receptor developed renal damage after protein overload, although to a lesser extent than wild-type littermates. To determine whether the difference in renal injury between BSA-treated AT1A+/+ and AT1A−/− mice could be due to the different BP levels achieved in the two strains, we performed additional long-term experiments. In these studies, we compared the development of renal damage after 11 wk of protein overload in AT1A−/− mice and in AT1A+/+ animals in which BP was intentionally maintained at comparable levels by treatment with the dihydropyridinic calcium channel blocker lacidipine. After 11 wk, body weight did not vary between AT1A+/+ and AT1A−/− BSA mice, reaching the average value of 34 ± 1 and 32 ± 1 g, respectively. Lacidipine treatment did not affect body weight (33 ± 1 g). During the 11-wk follow-up, SBP was significantly lower in lacidipine-treated AT1A+/+ BSA mice than in untreated AT1A+/+ BSA.
controls (Figure 5). In AT1A+/+/BSA mice, lacidipine reduced SBP to an extent that was comparable to the protein-overloaded AT1A+/− mice (Figure 5). The reduced BP in the AT1A+/+/BSA animals that were given lacidipine was associated with a reduction of urinary protein excretion rate to levels that were very comparable to those of AT1A+/− mice (Figure 6). Urinary protein excretion in AT1A+/+ and AT1A+/− mice that received saline and were followed for the same period was negligible. Urine output was higher in AT1A+/− mice as compared with AT1A+/+ mice that were given saline (1.8 ± 0.2 versus 1.0 ± 0.1 ml/24 h; P < 0.01). After BSA, a threefold increase in diuresis was observed in both groups of mice (AT1A+/−, 5.7 ± 0.4; AT1A+/+, 3.3 ± 0.4 ml/24 h; P < 0.01). Lacidipine did not further affect the urine output (3.5 ± 0.3 ml/24 h).

Values of left kidney weight are reported in Table 1. Kidney weights were comparable in AT1A+/+ mice that were given saline. After protein overload, kidney weight increased in both groups of mice, although to a different extent. Values of AT1A+/+BSA mice were higher than those of AT1A+/− mice (P < 0.01). Lacidipine mildly but significantly reduced kidney hypertrophy in AT1A+/+BSA mice (P < 0.05).

AT1A+/− mice as compared with AT1A+/+ mice that were given saline (1.8 ± 0.2 versus 1.0 ± 0.1 ml/24 h; P < 0.01). After BSA, a threefold increase in diuresis was observed in both groups of mice (AT1A+/−, 5.7 ± 0.4; AT1A+/+, 3.3 ± 0.4 ml/24 h; P < 0.01). Lacidipine did not further affect the urine output (3.5 ± 0.3 ml/24 h).

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Figure 3. Renal expression of endothelin-1 (ET-1) mRNA (a) and urinary excretion of ET-1 (b) at 4 wk in AT1A+/+ mice (n = 6), AT1A+/− mice (n = 6), and mice that received daily intraperitoneal injections of BSA (10 mg/g; AT1A+/+BSA, n = 11; AT1A+/−BSA, n = 7). Data are mean ± SEM. *P < 0.05, **P < 0.01 versus AT1A+/+; °P < 0.05, °°P < 0.01 versus AT1A+/−.

Figure 4. Photomicrographs showing the localization of ET-1 protein at 4 wk in sections of renal cortex from AT1A+/+ and AT1A+/− mice that were given saline (A and B) or daily injections of BSA (AT1A+/+BSA [C] and AT1A+/−BSA [D]. No specific signals were obtained in negative control (E). Magnification, ×100.

Figure 5. SBP at 11 wk in AT1A+/+ mice (n = 6), AT1A+/− mice (n = 6), and mice that received daily intraperitoneal injections of BSA (10 mg/g; AT1A+/+BSA, n = 11; AT1A+/+BSA+lacidipine, n = 7; AT1A+/−BSA, n = 7). Data are mean ± SEM. *P < 0.01 versus AT1A+/+; °P < 0.01 versus AT1A+/−.
Histologic examination of renal specimens at the end of the 11-wk follow-up showed marked glomerulosclerosis, interstitial inflammation, and tubulointerstitial injury in untreated AT1A+/+ BSA mice (Figure 7C). Results of semiquantitative analysis are shown in Table 1. Lacidipine treatment of AT1A+/+ BSA mice numerically reduced the percentage of glomeruli with focal and segmental sclerosis and the number of monocytes/macrophages infiltrating the interstitium and lowered tubulointerstitial damage (Figure 7D, Table 1). These morphologic changes were of comparable severity with respect to those found in AT1A−/− BSA mice (Figure 7E, Table 1). No evidence of significant glomerular and tubulointerstitial damage was found in AT1A+/+ and AT1A−/− animals that received saline at the end of the 11-wk follow-up (Figure 7, A and B, Table 1).

**Table 1.** Glomerular and tubular changes and monocyte/macrophage accumulation in the renal interstitium at 11 weeks

<table>
<thead>
<tr>
<th>AT1A+/+ saline</th>
<th>AT1A−/− saline</th>
<th>AT1A+/+ BSA</th>
<th>AT1A+/+ BSA + lacidipine</th>
<th>AT1A−/− BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (mg)</td>
<td>284 ± 31</td>
<td>311 ± 43</td>
<td>434 ± 13</td>
<td>392 ± 10c,d</td>
</tr>
<tr>
<td>GS (%)</td>
<td>0.2 ± 0.2</td>
<td>0</td>
<td>14 ± 5b</td>
<td>7 ± 1b</td>
</tr>
<tr>
<td>Tubular Damage</td>
<td>0.2 ± 0.2</td>
<td>0</td>
<td>1.4 ± 0.2b</td>
<td>1.2 ± 0.2b</td>
</tr>
<tr>
<td>(Score)</td>
<td></td>
<td></td>
<td></td>
<td>8 ± 2b</td>
</tr>
<tr>
<td>F4/80+ Cells (Cells/HPF)</td>
<td>0</td>
<td>0</td>
<td>27.2 ± 2.8b</td>
<td>16.0 ± 2.2b</td>
</tr>
</tbody>
</table>

*Values are mean ± SEM at 11 wk. HPF, high-power field; GS, glomerulosclerosis.

**Discussion**

In experimental and human proteinuric nephropathies, drugs that inhibit the RAS protect against renal function deterioration better than other antihypertensive agents (2). Whether such effect was due to the peculiar property of Ang II inhibitors of reducing excess protein ultrafiltration and its deleterious consequences or to the reduction of the proinflammatory and fibrogenic effects of Ang II is still a matter of debate. Mice with targeted disruption of the AT1A receptor gene have been instrumental here to dissect the relative contribution of proteinuria and Ang II, widely recognized as major determinants of renal disease progression. Data showed that targeted disruption of the AT1A receptor did not protect the kidney against renal functional and structural damage consequent to protein overload. Specifically, AT1A−/− mice acquired a renal phenotype of proteinuria and renal glomerular and tubulointerstitial lesions reminiscent of progressive nephropathies in humans. These findings can be taken to indicate that protein trafficking per se might be deleterious to the kidney independent of Ang II. In vitro studies and in vivo models of proteinuric renal diseases (26) indicate that reabsorption of filtered proteins activates the proximal tubular epithelium to generate...
vasoactive and inflammatory substances, including ET-1 (24) and chemokines (26–29), that are secreted toward the basolateral compartment of the proximal tubular cells, instigating an interstitial inflammatory reaction (26).

Reduced susceptibility to kidney damage in mice that lack AT_1A receptor could have been the consequence of lower systemic BP levels in AT_1A^{−/−} vis à vis AT_1A^{+/+} mice (14). A strong graded relation exists between BP and the severity of renal lesions in experimental animals independent from the underlying disease. Such relation has also been found in various renal diseases as highlighted in a recent pooled analysis of the major clinical studies (30). However, tight BP control uniformly slows disease progression and reduces injury. Of interest, in this context, data in humans have been provided recently to indicate that further lowering of BP as compared with previous guidelines is uniformly renoprotective (30,31). Because increases in BP levels in nephropathies translate in systemic BP levels in AT_1A deficient mice could be attributable to changes of those receptors. Severally possible that the renal pathology observed in AT_1A deficient and wild-type BSA-overloaded AT_1A^{+/+} mice, whose BP was kept lower by administration of the calcium channel blocker lacidipine throughout the experiment. Dihydropyridinic calcium antagonists are effective in lowering BP without interfering with the size-selective properties of the glomerular basement membrane (33). Reducing BP of AT_1A^{+/+}BSA mice to levels comparable to AT_1A^{−/−}BSA mice resulted in comparable values of protein excretion rate, similar degree of glomerular and tubulointerstitial lesions, and same amount of F4/80-positive cells infiltrating the kidney. Whether this last effect could be due to an anti-inflammatory response induced by lacidipine as it occurred in an Ang II–dependent transgenic rat model (34) could not be completely ruled out. Despite that no compensatory upregulation has been observed in the expression of AT_1B or AT_2 receptors in the mouse line here used (13), it is theoretically possible that the renal pathology observed in AT_1A^{−/−} mice could be attributable to changes of those receptors. Several hurdles make it unfeasible to explore the contribution of the AT_1B receptor’s residual activity in the renal phenotype of overload proteinuria. Mutant strains of mice that lack both AT_1A and AT_1B receptors have significantly reduced postnatal survival and spontaneously develop renal pathology that could confound the effect of overload proteinuria (13). Furthermore, blockade of AT_1B through an AT_1 receptor antagonist could not be of help in this setting because of the proteinuria-lowering effect of Ang II blockers. A number of studies have documented that signaling through AT_2 receptor results in vasodilation through a direct or bradykinin-induced stimulation of nitric oxide (35) and mediates antiproliferative responses and apoptosis (36,37). In line with the latter effect are data showing that AT_2 receptor null mice display deficient apoptosis and increased fibrosis after ureteral obstruction (38). By contrast, overexpression of AT_2 receptors protected mice with renal mass reduction from the development of renal lesions (39). The similarity of the beneficial effect of ACE inhibitors and AT_1 receptor antagonists in experimental models and clinical studies would further support the notion that AT_2 receptor stimulation does not contribute to the pathogenesis of renal disease. Other recent studies have challenged the view of AT_2 as a “protective” receptor (40) and provided evidence that the growth stimulatory and proinflammatory effects of Ang II also may be transduced by AT_2, which induces glomerular cell proliferation and monocyte/macrophage infiltration, albeit to a lower extent than AT_1 receptor (41,42). This observation together with the evidence of AT_2 upregulation in protein-overload proteinuria (43) would offer the rationale for studies with specific AT_2 antagonists. However, the recent demonstration that these compounds had some antiproteinuric effect in rats with progressive renal injury (42) would render difficult their use to discriminate the role of Ang II through AT_2 receptors and proteinuria. It would have been nice to explore the potential role for AT_2 receptors. However, because experiments with specific antagonists are unlikely to provide a clearcut answer to the question of a possible role for the reasons described above, we are forced to conclude that the present study cannot rule out the possibility that Ang II still induces renal damage in AT_1A^{−/−} mice via AT_2 type receptors. Renal lesions that develop in animals with targeted disruption of AT_1A receptor should not be taken to disregard the additional role of Ang II in chronic renal disease. Experimental and human studies have convincingly documented that Ang II has a central role in glomerular hemodynamic changes associated with progressive renal injury (44). Nonhemodynamic effects of Ang II might be important as well (45). Those include renal cell proliferation, exuberant TGF-β synthesis, and activation of infiltrating macrophages. The additional role of Ang II is in keeping with findings here that renal lesions were more severe in AT_1A^{+/+} mice. Proteinuria was also more severe in AT_1A^{+/+} mice than in AT_1A^{−/−} mice. More proteinuria in AT_1A^{+/+} mice that were exposed to protein overload reflects size-selective dysfunction possibly involving podocytes and podocyte slit diaphragm architecture. Podocytes—the critical component of barrier size selectivity (46)—express on their surface specific receptors for Ang II that govern cytoskeletal organization upon stimulation. This is consistent with data in isolated perfused kidney that infusion of Ang II enhances filtration of large macromolecules and promotes proteinuria, and these effects are prevented by losartan (47). In vivo in animals and humans with proteinuric nephropathies, drugs that inhibit binding of Ang II to AT_1 receptors ameliorate glomerular size-selective function, normalize proteinuria, and retard disease progression (48,49).

An additional finding of the present study was that both intrarenal gene expression and synthesis of ET-1, a mediator of tubulointerstitial damage, were upregulated at comparable levels in AT_1A deficient and wild-type BSA-overloaded mice. Changes in renal ET-1 expression and synthesis are similar to those reported in the study by Suzuki et al. (8) that was aimed at examining the potential interrelationship between Ang II and ET-1 in the tubulointerstitial damage in AT_1A deficient mice and pointing out the higher-than-normal expression of ET-1 in tubular epithelial cells. Notably, the administration of an ET-1
receptor antagonist to AT$_{1A}$ deficient mice effectively reduced the interstitial cell infiltrates without affecting proteinuria (8). Phenotypic changes of proximal tubular cells triggered by excessive protein reabsorption include the induction of ET-1 synthetic pathway (24) to the extent that ACE inhibitors by normalizing proteinuria reduced excessive ET-1 synthesis in renal tubuli. Whether changes in ET-1 synthesis were a direct effect of protein overload or rather a consequence of Ang II, a major stimulus for ET-1 in renal cells (8,50), remains elusive so far. Findings here of comparable expression of ET-1 in AT$_{1A}^-/-$ and AT$_{1A}^{+/+}$ mice after protein overload indicate that proteinuria rather than Ang II triggers renal ET-1 upregulation in progressive nephropathies.

In summary, our findings indicate that proteinuria might be a player for renal damage even independent from Ang II. These observations together with previous findings in humans that early changes in proteinuria predict long-term disease progression independent of BP control (51) provide a clear rationale for specifically targeting proteinuria in renoprotective pharmacologic interventions.

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References
tion, and tissue factor in angiotensin II-induced end-organ damage. Hypertension 36: 282–290, 2000