Podocytes play an indispensable role as a filtration barrier for macromolecules in the glomerulus. Damage of podocytes is a key step triggering the progression of glomerulosclerosis. A large volume of evidence indicates that angiotensin (Ang) II acting on the Ang II type 1 receptor (AT₁) plays important roles in this process. Blockade of Ang II synthesis with Ang I–converting enzyme (ACE) inhibitors or of Ang II action with AT₁ receptor blocker (ARB) is a key to the protective effect of pharmacological Ang II blockade. In fact, several lines of evidence indicate that Ang II has direct cellular effects on podocytes. Cultured mouse podocytes express mRNA and protein for AT₁, and Ang II depolarizes and increases intracellular Ca²⁺. In cultured podocytes, Ang II, via AT₁, increases α3(IV) collagen and desmin expression and suppressed nephrin and podocin mRNA. Studies conducted thus far collectively indicate that Ang II is involved in triggering, enhancing, and expanding podocyte injury and in the progression of glomerular injury toward sclerosis through mechanisms beyond its effect on systemic blood pressure.

Because podocyte injury along with proteinuria ubiquitously precedes progressive development of glomerulosclerosis, and because Ang II inhibition attenuates podocyte damage and progressive glomerulosclerosis, it appears reasonable to speculate that inhibition of AT₁ on podocytes is the key to the protective effect of pharmacological Ang II blockade. A part of this study was presented in an abstract form at the annual meeting of the American Society of Nephrology on November 6, 2008 in Philadelphia, Pa.
vascular endothelial growth factor decreases nephrin, heparan sulfate proteoglycans, and α-actin; 4: augments reactive oxygen species production; and induces redistribution of zona occludens 1 and reorganization of F-actin cytoskeleton. More directly, transgenic rats overexpressing the AT1 receptor selectively in podocytes develop glomerulosclerosis. These data are consistent with the notion that the beneficial effect of Ang II blockade on glomerulosclerosis is attributed to its direct inhibitory effects on Ang II action on podocytes.

Earlier, we established a transgenic mouse line (NEP25) that expresses human (h) CD25 (ie, interleukin 2 receptor) selectively on podocytes. Because hCD25 does not react with mouse interleukin 2 ligand, it is highly unlikely that expression of hCD25, per se, affects podocyte function, including Ang II signaling. By injecting an hCD25-targeted recombinant immunotoxin, anti-Tac(Fv)-PE38 (LMB2), podocyte-selective injury can be induced in NEP25 mice. LMB2 is a recombinant chimeric protein composed of PE38 (a mutant form of pseudomonas exotoxin A) and the Fv domain of monoclonal antiCD25 antibody. LMB2 (mass: 63 kDa) can cross the glomerular basement membrane, and its half-life in the circulation is 35 minutes in mice. After a single injection of LMB2 (at 0.625 ng/g of body weight [BW]), NEP25 mice develop moderate proteinuria, which peaks 1 to 2 weeks after the injection and gradually decreases. Within 2 weeks, NEP25 mice have minor podocyte injury. After 3 weeks, they show progressive damage of podocytes and other glomerular cells and develop focal segmental glomerulosclerosis.

To clarify the role of Ang II on podocytes during the progression of glomerulosclerosis, we generated podocyte-specific AT1−/− mice. Unlike humans, mice have 2 AT1 receptor genes, Agt1a and Agt1b, each encoding AT,A and AT,B receptor subtype, respectively. In the mouse kidney, >99% of AT1 mRNA is derived from Agt1a, and Agt1a inactivation does not lead to activation of Agt1b. We, therefore, used podocyte-specific Agt1a null-mutant mice.

Methods

The Tokai University Animal Experimentation Committee approved the protocol, in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Generation of Podocyte-Specific Agt1a Null-Mutant Mice

Detailed methods for generation of Agt1a−/− mice (C57BL/6 background) are described in the online Data Supplement (please see http://hyper.ahajournals.org.) Nephrin-Cre mice were reported previously. In the present study, line 10 of Nephrin-Cre mice were used. They were backcrossed with the C57BL/6 strain >3 times and used for mating with Agt1a−/− mice. To induce podocyte-specific injury, mice carrying Agt1a−/− and Nephrin-Cre were further mated with NEP25 mice on the C57BL/6 genetic background.

Determination of Genotype of Podocytes

From mice carrying the Agt1a−/−/Cre+ /TRE-SV40T/podocin-rtTA/ROSA26loxP or Agt1a+/+ /Cre+ /TRE-SV40T/podocin-rtTA/ROSA26loxP genotype, glomeruli were isolated by perfusing with Dynabeads (Dynal ASA). Glomeruli were cultured on laminin-coated dishes in the presence of doxycycline (1 μg/mL) for

Figure 1. Verification of Agt1a disruption in the podocytes of Agt1a−/−/Cre+ mice. A, PCR for the genotyping of Agt1a. In wild-type Agt1a, the primer pair AT6 and AT7 generated a 300-bp band. The primer pair AT5 and AT7 did not amplify DNA. In Agt1a−/−, the primer pair AT6 and AT7 generated a 366-bp band, whereas the primer pair AT5 and AT7 did not amplify DNA. In Agt1a−/−, the primer pair AT5 and AT7 generated a 213-bp band. The portion recognized by AT6 is removed from the Agt1a−/− genome. B, Representative PCR result. Lane 1, DNA size markers. Lane 2, ES cell DNA carrying Agt1a−/+. Lane 3, ES cell DNA carrying Agt1a+/−. Lane 4, Tail DNA carrying Agt1a−/− (wild type). Lane 5, Tail DNA carrying Agt1a−/+. Lanes 6 and 7, LacZ-tagged podocyte clones from mice carrying Agt1a−/−/Cre+/TRE-SV40T/podocin-rtTA/ROSA26loxP. Both show the Agt1a−/− genotype. Other 6 clones (not shown) similarly showed the Agt1a−/− genotype. Lanes 8 to 12, LacZ-tagged podocyte clones from mice carrying Agt1a−/−/Cre+/TRE-SV40T/podocin-rtTA/ROSA26loxP. All show the Agt1a−/− genotype. Thirteen (87%) of 15 clones examined showed the Agt1a−/− genotype.
PCR was carried out using primers GCATCATCTTTGTGGTGGG and GAAGAAGAACGACATCGGCC, which are common to both AT1A and AT1B sequences. Only the PCR product derived from AT1A mRNA has an EcoRI site. Therefore, EcoRI-cleaved and uncleaved bands represent AT1A and AT1B mRNA, respectively. AT1A and AT1B mRNA were also quantified by real-time RT-PCR. TaqMan Primer Probe sets for Agtr1a, Agtr1b, and 18S RNA were used with Applied Biosystems 7300 Real-Time PCR systems. The amplification efficiency for AT1A and AT1B was 51% to 75%, or represents the sclerotic area involving 0%, 1% to 25%, 26% to 50%, respectively, in an outside laboratory (SRL). Concentration of total protein and creatinine in the urine were determined by the pyrogallol red and enzymatic methods, respectively, in an outside laboratory (SRL). Concentration of albumin in the urine was determined by a standard template containing an equal molar of AT1A and AT1B cDNAs.

Real-time RT-PCR was also performed in RNA extracted from primary cultured podocytes after the first and second passages. Primary cultured podocytes were obtained from wild-type mice (n=3), as described previously.27

Experimental Protocol of Immunotoxin-Induced Nephropathy

To study the effect of podocyte-specific AT1 inactivation, 5 female and 8 male mice carrying Agtr1aloxP/loxP/Cre(−)NPE25 and 10 female and 8 male mice carrying Agtr1aloxP/loxP/Cre(+)NPE25 (3 to 7 months of age) were used. Twenty-four–hour urine was collected before and after LMB2 injection. They were euthanized 28 days after LMB2 injection.

To study the effect of losartan, an AT1 receptor blocker, 8 female NPE25 mice with a C57BL/6 genetic background (5 months of age) were treated with losartan (0.5 g/L, in drinking water, NEP25) mice with a C57BL/6 genetic background (5 months of age) were used. Twenty-four–hour urine was collected before and after LMB2 injection. They were euthanized 28 days after LMB2 injection.

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Statistical Analysis

Results are expressed as mean±SE. Student t test was used to analyze the difference between 2 groups in blood pressure. One-way ANOVA was used to compare blood pressure among 3 groups. Albumin/creatinine ratio was measured repeatedly, and global test assessing the between-group effect was performed through multivariate ANOVA to prevent inflation of type I error through multiple comparisons. When overall effect was detected with multivariate ANOVA, Student t test was performed at individual time points. Comparison of sclerosis index and nephrin index was performed by the Mann-Whitney U test. Values were regarded as significant at 2-sided P<0.05.

Results

Generation of Agtr1aloxP/loxP Mouse

To investigate the direct effect of Ang II on podocytes, we generated podocyte-specific Agtr1a-null mutant mice. Using homologous recombination in ES cells, we established mutant mice carrying Agtr1aloxPloxP, in which 2 loxP sites were inserted at the upstream and the lower stream to the coding exon of Agtr1a (Figure S1, please see the online Data Supplement at http://hyper.ahajournals.org).

Homozogous (Agtr1aloxPloxP) and heterozygous (Agtr1aloxP/+) mice showed no apparent abnormal phenotype. Northern blotting analysis revealed that Agtr1aloxPloxP and Agtr1aloxP/+ mice similarly expressed AT1A mRNA in the kidney compared with wild-type mice (data not shown). Systolic blood pressures were 97±13 and 105±8 mm Hg in Agtr1aloxPloxP and Agtr1aloxP/+ mice, respectively, similar to that in wild-type littermates (100±12 mm Hg). Agtr1aloxPloxP showed normal renal morphology. These data confirm that the insertion of the loxP sequences did not disturb the expression and the function of AT1A mRNA.

Confirmation of Agtr1a Disruption in Podocytes of Agtr1aloxPloxP/Cre(+) Mice

The Agtr1aloxPloxP line was mated with a Nephrin-Cre line, which expresses Cre recombinase selectively in podocytes. Previously, we tested the efficiency of Cre-mediated recombination in podocytes by mating Nephrin-Cre mice with ROSA26loxP, a tester strain. One-hundred percent of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP, a tester strain. One-hundred percent of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP, a tester strain. One-hundred percent of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP, a tester strain. One-hundred percent of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP, a tester strain. One-hundred percent of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP, a tester strain.

Urinalysis

Twenty-four–hour urine specimens were collected using metabolic cages. Concentrations of total protein and creatinine in the urine were determined by the pyrogallol red and enzymatic methods, respectively, in an outside laboratory (SRL). Concentration of albumin in the urine was determined with an ELISA kit (Albuwell M).

Morphological Analysis

Glomerulosclerosis was evaluated in PAS-stained paraffin sections (2-μm thick). Each glomerulus was graded on a 0 to 4 scale, which represents the sclerotic area involving 0%, 1% to 25%, 26% to 50%, 51% to 75%, or >75% of the glomerulus. Scores for all of the glomeruli on a section were averaged and defined as the sclerosis index for each mouse.

For evaluating podocyte injury, paraffin sections were stained for nephrin using guinea pig polyclonal antibody (GP-N2, Progen). For semiquantification of nephrin staining, each quadrant of each glomerulus was scored as 0 (no staining), 1 (diminished), or 2 (normal), with total glomerular score range calculated from 0 (complete loss) to 8 (normal). Scores for all of the glomeruli on a section for each mouse (>80) were averaged and defined as the nephrin index.
Podocyte-selective AT1 knockout mice, *Basal Phenotype of Agtr1a* mice showed that all of the lacZ-positive clones had the *Agtr1a*\textsuperscript{loxP}/\textsuperscript{loxP}/\textsuperscript{Cre} genotype. Similar analysis in 8 lacZ-positive colonies from *Agtr1a*\textsuperscript{loxP}/\textsuperscript{loxP}/\textsuperscript{Cre} mice showed only deleted allele *Agtr1a*\textsuperscript{loxP}/\textsuperscript{loxP}/\textsuperscript{Cre} in injury.

Figure 2. Effect of podocyte-specific AT1 deletion on glomerular injury. *Agtr1a*\textsuperscript{loxP}/\textsuperscript{loxP}/\textsuperscript{Cre} mice were injected with LMB2 (0.625 ng/g of BW). There was no significant difference in urinary albumin:creatinine ratio between the 2 types of mice. Without LMB2, both types of mice showed no sclerosis or podocyte damage, with a sclerosis index of 0 and a nephrin index of 8.

15 lacZ-positive clones examined showed only deleted allele (*Agtr1a*\textsuperscript{-}; Figure 1). Two lacZ-positive colonies showed both *Agtr1a*\textsuperscript{loxP} and *Agtr1a*\textsuperscript{-}, indicating that they were heterozygote (*Agtr1a*\textsuperscript{loxP/loxP}). LacZ-negative cobblestone-like cells often grew even without doxycycline. PCR analysis revealed that all 3 such colonies examined had the *Agtr1a*\textsuperscript{loxP/loxP/Cre} genotype. Similar analysis in 8 lacZ-positive colonies from *Agtr1a*\textsuperscript{loxP}/\textsuperscript{Cre}/\textsuperscript{TRE-SV40T/podocin-rtTA/ROSA26 loxP} mice showed that all of the lacZ-positive clones had the *Agtr1a*\textsuperscript{-/loxP/loxP/Cre} genotype. These confirmed that Cre-mediated recombination of *Agtr1a*\textsuperscript{loxP} occurs efficiently in podocytes, and most podocytes in *Agtr1a*\textsuperscript{loxP/loxP/Cre} mice were indeed null mutated for *Agtr1a*.

**Basal Phenotype of Agtr1a\textsuperscript{loxP/loxP/Cre}(+) Mice**

Podocyte-selective AT1 knockout mice, *Agtr1a*\textsuperscript{loxP/loxP/Cre}(+), showed no apparent abnormal phenotype in a basal condition. Thus, at 4 months of age, the urinary albumin:creatinine ratio in *Agtr1a*\textsuperscript{loxP/loxP/Cre}(+) mice was not different from that in control *Agtr1a*\textsuperscript{loxP/loxP/Cre}(-) mice, either male (0.17±0.02, n=8, versus 0.14±0.02, n=10) or female (0.05±0.01, n=8, versus 0.05±0.02, n=10). Systolic blood pressure measured at 4 months of age in *Agtr1a*\textsuperscript{loxP/loxP/Cre}(+) mice was not different from that in *Agtr1a*\textsuperscript{loxP/loxP/Cre}(-) mice (118.0±3.2 versus 113.4±5.0 mm Hg; each n=6). Renal histology and nephrin staining in *Agtr1a*\textsuperscript{loxP/loxP/Cre}(+) mice were normal over a range from age 1 to 12 months (data not shown).

To determine the relative amount of AT1A and AT1B mRNA, RT-PCR was performed in glomerular RNA from *Agtr1a*\textsuperscript{loxP/loxP/Cre}(+) and *Agtr1a*\textsuperscript{loxP/loxP/Cre}(-) mice using primers common to AT1A and AT1B sequences following digestion with EcoRI, which is specific to AT1A. AT1B mRNA was undetectable in glomeruli of either type of mice with this method. Real-time RT-PCR revealed that AT1B mRNA was detectable in the glomerulus, but the quantity was <0.5% (0.0% to 0.5%; n=4) of that of AT1A in either *Agtr1a*\textsuperscript{loxP/loxP/Cre}(+) or *Agtr1a*\textsuperscript{loxP/loxP/Cre}(-) mice. Induction of podocyte injury by LMB2, which is shown below, did not enhance AT1B mRNA. In addition, AT1A mRNA was detectable in primary cultured podocytes by RT-PCR, but AT1B mRNA was undetectable.

**Effect of Podocyte-Specific Inactivation of AT1 on Progression of Glomerulosclerosis**

To examine whether podocyte-specific deletion of AT1 receptor can slow the progression of glomerulosclerosis, we mated *Agtr1a*\textsuperscript{loxP/loxP/Cre}(+) mice with the NEP25 line, generating *Agtr1a*\textsuperscript{loxP/loxP/Cre}(+)/NEP25 and *Agtr1a*\textsuperscript{loxP/loxP/Cre}(+)/NEP25 mice. Without LMB2, both types of mice showed no proteinuria (Figure 2A, before LMB2), and renal morphology and nephrin staining were normal (Figure 3A and D) with a sclerosis index of 0 and a nephrin score of 8. After injection of 0.625 ng/g of BW of LMB2, both types of mice showed moderate proteinuria, which peaked 2 weeks after the injection, with no statistical difference at any time point (Figure 2A).

The degree of focal segmental glomerulosclerosis was similar in both mouse groups at 4 weeks. *Agtr1a*\textsuperscript{loxP/loxP/Cre}(+)}...
Cre(+) /NEP25 had a sclerosis index of 0.65±0.16, which was not statistically different from that of Agtr1aloxP/loxP/Cre(−)/NEP25 mice, at 0.82±0.27 (Figures 2B and 3). Both types of mice had glomeruli with similarly diminished nephrin staining. The nephrin index in Agtr1aloxP/loxP/Cre(+) /NEP25 was, on average, 5.78±0.45, which was not statistically different from that of Agtr1aloxP/loxP/Cre(−)/NEP25 mice, at 5.63±0.58 (Figures 2C and 3). Thus, podocyte-specific inactivation of AT1 showed no impact on the progression of glomerulosclerosis triggered by podocyte injury.

**ARB Attenuates Progression of Glomerulosclerosis in NEP25 Model**

We next examined whether ARB can protect against the glomerular injury in NEP25 transgenic mice, which progressively develops after the injection of LMB2 (0.625 ng/g of BW). The ARB-treated mice showed significantly lower systolic blood pressure than the control mice without treatment (66±6 versus 101±5 mm Hg). NEP25 mice without ARB showed moderate proteinuria with a marked decrease in ARB NEP25 (urinary protein:creatinine ratio: 66.6±20.7 versus 22.8±10.3 mg/mg) 7 days after LMB2 injection (Figure 4A). Twenty-one days after injection, NEP25 mice showed focal segmental sclerosis with a sclerosis index averaging 0.83±0.36, demonstrating marked protection in ARB NEP25 mice (0.01±0.01; Figure 4B and 5).

Podocyte injury, assessed by nephrin staining, was significantly attenuated in NEP25 versus ARB NEP25 (nephrin staining index: 5.61±0.76 versus 7.96±0.01, to 8 scale; Figures 4C and 5). Similar protection was observed in Agtr1aloxP/loxP/Cre(+) /NEP25 mice treated with ARB (Figure 6). Agtr1aloxP/loxP/Cre(−)/NEP25 mice treated with ARB starting after LMB2 injection (Figure S2), and NEP25 mice with captopril (Figure S3) but not with hydralazine (Figure S4).

**Effect of Podocyte-Specific Inactivation of AT1 on Ang II–Induced Microalbuminuria**

We next examined the effect of podocyte-specific inactivation of AT1, on microalbuminuria induced by Ang II infusion. A pressor dose of Ang II (1000 ng/kg per minute) was continuously infused for 14 days using miniosmotic pumps in Agtr1aloxP/loxP/Cre(−) and Agtr1aloxP/loxP/Cre(+) mice. Systolic blood pressure was similarly elevated in both groups (149.3±11.2 versus 143.5±14.3, respectively). As shown in Figure 7, both strains of mice showed a similar degree of microalbuminuria at all of the time points examined. Under

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**Figure 4.** Effect of ARB treatment on NEP25 mice. NEP25 mice were injected with LMB2 (0.625 ng/g of BW). Mice with ARB treatment that started before LMB2 injection (■) showed significantly attenuated urinary total protein:creatinine ratio (U-Pro/Cr; A), glomerulosclerosis (B), and downregulation of nephrin (C) when compared with those in control mice without ARB (□). Without LMB2, NEP25 mice showed no sclerosis or podocyte damage with a sclerosis index of 0 and a nephrin index of 8.

**Figure 5.** Representative pictures of NEP25 mice with or without ARB. NEP25 mice 21 days after LMB2 without ARB treatment show severe glomerulosclerosis (A) and a remarkable decrease in nephrin staining (C, arrows). In ARB-treated NEP25 mice 21 days after LMB2, glomerular injury (B) and nephrin staining (D) are remarkably improved. A through C, periodic acid Schiff, ×200; D through F, antinephrin, ×400.

**Figure 6.** Effect of ARB treatment on Agtr1aloxP/loxP/Cre(+) /NEP25 mice. Agtr1aloxP/loxP/Cre(+) /NEP25 mice were injected with LMB2 (0.625 ng/g of BW). Mice with ARB treatment (■) showed significantly attenuated urinary albumin:creatinine ratio (ACR; A), glomerulosclerosis (B), and downregulation of nephrin (C) when compared with those in control mice without ARB (□). P<0.05.
this experimental condition, no mouse in these groups showed glomerular sclerosis or downregulation of nephrin staining.

Discussion

In the present study, blockage of Ang II, by either an ARB or ACE inhibitor, attenuated proteinuria, podocyte injury, and glomerulosclerosis in the NEP25 model in a fashion similar to those of other models for podocyte injury. Treatment with hydralazine showed no renal protective effect, although it effectively decreased blood pressure, indicating that the protective effect of Ang II blockage is independent of its systemic blood pressure-lowering effect. Because an ACE inhibitor was also effective, the beneficial effect of ARB is ascribed to inhibition of AT1, not to an activation of non-AT1 Ang II receptor(s).

As discussed earlier, podocytes in vitro, as well as in vivo, have been shown to express functional AT1.13,14 Moreover, transgenic rats overexpressing the AT1 receptor selectively in podocytes showed progressive increase in albuminuria and pseudocyst formation in podocytes, which were followed by the development of glomerulosclerosis.23 The present study failed to show that podocyte-specific AT1 inactivation has any impact on baseline or Ang II-induced proteinuria. One might consider the possibility that this apparent discrepancy is because of a difference in the duration and/or magnitude of AT1 stimulation. In this regard, our study on podocyte-specific AT1 knockout mice indicates that the microalbuminuria induced by short-term (2-week) Ang II infusion does not reflect its local effect on podocytes. This, in turn, points to the notion that an event occurring in nonpodocyte cells leads to alteration in the sieving function of the glomerulus.

Of importance, the present study convincingly demonstrated that an AT1 blocker protects podocytes primarily by a mechanism independent of its inhibitory effect on the AT1 on podocytes. Ang II can increase glomerular capillary ultrafiltration pressure by increasing efferent arteriolar resistance and/or by lowering afferent arteriolar resistance.28,29 This leads to an enhancement of leakage of macromolecules through the glomerular capillary wall.30 A variety of studies have demonstrated association between the glomerular capillary pressure and the progression of glomerulosclerosis. Although a molecular mechanism for this connection has yet to be established, the present study is consistent with the view that the capacity of an ARB to decrease glomerular capillary pressure contributes to the protective effect of an ARB in this NEP25 model. Theoretically, this notion can be verified by a study on efferent arteriole-specific AT1 knockout mice. However, no promoter segment is currently known that can drive efferent arteriole-specific expression of the Cre gene.

LMB2 inhibits protein synthesis by inactivating elongation factor 2 in targeted podocytes. One may, therefore, consider the possibility that the lack of a beneficial effect of podocyte-specific AT1 inactivation is ascribed to suppression of the AT1 protein in podocytes of the control NEP25 mice (Agtr1aloxP/loxP/Cre(-)/NEP25). Although we did not quantify the amount of AT1 protein in podocytes, the dose of LMB2 in this study did not decrease any of the other proteins examined thus far, including nephrin, Wilms tumor 1, vascular endothelial growth factor, and synaptopodin, at a week after injection in NEP25 mice. In addition, ARB was also equally effective on control NEP25 mice given LMB2.

Unlike humans, mice have AT1B, another subtype AT1 receptor. The content of AT1B mRNA was <0.5% of AT1A mRNA in whole kidney.24–31 Recently, Crowley et al32 reported that AT1B mRNA is concentrated in podocytes and that the AT1B receptor may be stimulated by increased Ang II ligand in whole body AT1A knockout mice. Our results of a lack of protective effect of podocyte-specific AT1A inactivation are not attributed to compensation by AT1B. Previously, no Ang II binding in the presence of an Ang II type 2 antagonist was detected in the kidney of AT1A knockout mice by binding autoradiography.33 In the present study, we found that AT1B mRNA was <0.5% of AT1A mRNA in the glomerulus and was not increased by podocyte-AT1A knockout or by LMB2 injection. In primary cultured podocytes, AT1A mRNA, but not AT1B mRNA, was detectable by RT-PCR. Moreover, podocyte-specific AT1A knockout mice had normal blood pressure; therefore, it is unlikely that significant upregulation occurred in renin or Ang II ligand.

The remaining possibilities for the beneficial effects of Ang II blockage include inhibition of AT1 on nonpodocyte cells within and outside the glomerulus. The latter includes zona glomerulosa of the adrenal gland, that is, via inhibition of aldosterone synthesis and release. Some actions of ARB unrelated to Ang II, such as antioxidant effect of ARBs and ACE inhibitors, may also be contributory.

Perspectives

The unique design of the present study reveals a novel concept, namely, therapeutic measures even when targeting only nonpodocyte cells directly can profoundly affect the process of glomerulosclerosis, including measures initially triggered by selective podocyte injury. This notion will broaden the options in designing therapeutic measures to disrupt the process of glomerulosclerosis.

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References


Angiotsin Receptor Blocker Protection Against Podocyte-Induced Sclerosis Is Podocyte Angiotsin II Type 1 Receptor-Independent
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ARB Protection Against Podocyte-Induced Sclerosis is Podocyte AT1-Independent.

Short title: Podocyte AT1 and Glomerulosclerosis

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Methods

Generation of Agtr1a\textsuperscript{loxP} mice

A targeting vector was constructed for conditional targeting of the Agtr1a gene (Figure S1). The vector contains 13 kb of intron 2, exon 3 (the coding exon) and 2.8 kb of 3’ flanking region. At the Bgl II site of intron 2, upstream of the coding exon, one loxP site was inserted. At the Pst I site, downstream of the coding exon, a neomycin resistant gene cassette (pgk-neo) flanked by two loxP sites was inserted. At the 3’ end of the vector, an expression cassette for herpes simplex virus thymidine kinase (pgk-tk) was connected. The resultant targeting vector was introduced into E14.1 cells by electroporation. 768 G418 resistant ES colonies were picked up and analyzed by Southern blot analysis. Fourteen clones (18%) had undergone homologous recombination. We selected one clone, expanded it, and used for further experiments. To delete pgk-neo, a Cre expression vector, pCre-Pac plasmid (Kurabo, Osaka, Japan), was transiently introduced into the ES clone by electroporation. 196 puromycin-resistant colonies were analyzed by Southern blot analysis. Twelve colonies had desired recombination, i.e., pgk-neo was deleted while Agtr1a coding exon was preserved. The loxP insertion was confirmed by PCR amplification and sequencing. This allele is designated as Agtr1a\textsuperscript{loxP}. One of the targeted ES cells was injected into C57BL/6 blastocysts. One of the 10 chimeric mice obtained showed germline transmission of Agtr1a\textsuperscript{loxP} when mated with C57BL/6 females. Genotyping for Agtr1a was performed by PCR using primers, TTCAGCCAGATCGAGGAGCGGAGG and CTAACCGTTGAAATAGCTGTCC, which generate 217 base pair (bp) band in wild-type Agtr1a and 251 bp band in Agtr1a\textsuperscript{loxP}. Heterozygous (Agtr1a\textsuperscript{loxP+/+}) mice were backcrossed with C57BL/6 strain more than 10 times before mating with Nephrin-Cre mice.

Results

Generation of podocyte-specific Agtr1a knockout mice

To generate podocyte-specific Agtr1a-null mutant mice, we established mutant mice carrying Agtr1a\textsuperscript{loxP}, in which two loxP sites were inserted before and after the coding exon of Agtr1a (Figure S1). Northern blotting analysis
revealed that Agtr1a<sup>loxP/loxP</sup> and Agtr1a<sup>loxP/+</sup> mice similarly expressed AT1A mRNA in the kidney compared to wild-type mice (data not shown). Systolic blood pressure was 97±13 and 103±8 mmHg in Agtr1a<sup>loxP/loxP</sup> and Agtr1a<sup>loxP/+</sup> mice, respectively, which was not different statistically from that in wild-type littermates (100±12 mmHg). Agtr1a<sup>loxP/loxP</sup> and Agtr1a<sup>loxP/+</sup> mice showed normal renal morphology. These data confirm that the insertion of the loxP sequences did not disturb the expression and the function of AT1A mRNA.

Then, the Agtr1a<sup>loxP/loxP</sup> line was mated with a Nephrin-Cre line, which expresses Cre recombinase selectively in podocytes. Previously, we tested efficiency of Cre-mediated recombination in podocytes by mating Nephrin-Cre mice with ROSA26loxP, a tester strain. 100% of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP. We next tested whether recombination occurs in similar efficiency in podocytes of Agtr1a<sup>loxP/loxP/Nephrin-Cre</sup> (Agtr1a<sup>loxP/loxP/Ce(+)</sup>) mice. Reliable anti-mouse AT1 antibodies are not available to us and we therefore cultured podocytes, and then cloned and determined the Agtr1a genotype by PCR. For this purpose, Agtr1a<sup>loxP/loxP/Ce(+)</sup> mice were mated with TRE-SV40T/podocin-rtTA mice, which express SV40 T antigen in podocytes in the presence of doxycycline. To mark the podocyte-lineage with lacZ, the mice were further mated with ROSA26loxP line.

Glomeruli obtained from Agtr1a<sup>loxP/loxP/Ce(+)/TRE-SV40T/podocin-rtTA/ROSA26loxP</sup> mice were cultured in the presence of doxycycline. Colonies, each stem from a single cell, were stained for lacZ. PCR analysis revealed that 13 out of 15 (87%) lacZ-positive clones examined showed only deleted allele (Agtr1a<sup>-</sup>)(Figure S2). Two lacZ positive colonies showed both Agtr1a<sup>loxP</sup> and Agtr1a<sup>-</sup>, indicating that they are heterozygote (Agtr1a<sup>loxP/loxP</sup>). LacZ-negative cobblestone-like cells often grew even without doxycycline. PCR analysis revealed that all three such colonies examined had Agtr1a<sup>loxP/loxP</sup> genotype. Similar analysis in eight lacZ positive colonies from Agtr1a<sup>loxP/+ /Cre(+)/TRE-SV40T/podocin-rtTA/ROSA26loxP</sup> showed that all lacZ-positive clones had Agtr1a<sup>+/+</sup> genotype. These confirmed that Cre-mediated recombination of Agtr1a<sup>loxP</sup> occurs efficiently in podocytes and most podocytes in Agtr1a<sup>loxP/loxP/Ce(+)</sup> mice were actually null-mutated for Agtr1a.
Effect of losartan treatment starting after the LMB2 injection on NEP25 mice

Female Agtr1aloxP/loxP/Cre(-)/NEP25 mice (2-8 months of age) were injected with LMB2 (0.625 ng/g BW). 12 mice were treated with losartan (0.5 g/L in drinking water) 12 hours after the injection of LMB2 until the end of the experiment. They were compared with the 12 age-sex-matched mice injected with LMB2 without losartan treatment. Age did not affect the severity of renal injury induced by LMB2. Mice were sacrificed 28 days after the injection.

Losartan treatment attenuated glomerular injury. Thus, 28 days after the LMB2 injection, urinary albumin/creatinine ratio was, on average, 0.63±0.17, in the losartan group, which was significantly lower than that in the control group, 31.25±9.99 (Figure S2). Glomerulosclerosis and downregulation of nephrin were attenuated in the losartan group. Sclerosis index in the losartan group was, on average, 0.64±0.17, which was significantly lower than that in the control group, 1.80±0.40 (Figure S2). Nephrin index in the losartan group was, on average, 6.09±0.46, which was significantly higher than that in the control group, 4.00±0.76 (Figure S2).

Effect of captopril treatment starting after the LMB2 injection on NEP25 mice

Six female NEP25 mice with C57BL/6 genetic background (4 months of age) were treated with an ACE inhibitor, captopril (0.5 g/l, in drinking water, approximately 25 ng/g BW) 12 hours after LMB2 injection until the end of the experiment. Six age-sex-matched NEP25 mice with LMB2 without captopril were used as controls. Mice were sacrificed 28 days after the injection.

Captopril treatment remarkably well protected glomeruli, with sclerosis index, 0.01±0.01 (vs. 0.35±0.09 in control) and nephrin index, 7.98±0.01 (vs. 7.14±0.22 in control) (Figure S3).

Effect of hydralazine treatment on NEP25 mice

Six female NEP25 mice with C57BL/6 genetic background (3 months of age) were treated with hydralazine (250mg/l, in drinking water,
approximately 12 ng/g BW) from 5 days before LMB2 injection until the end of the experiment. Six age-sex-matched NEP25 mice injected with LMB2 without hydralazine were used as controls. Mice were sacrificed 21 days after the injection.

Systolic blood pressure was measured by tail cuff method two days before the LMB2 injection. The hydralazine-treated mice showed significantly lower systolic blood pressure than the control mice without treatment (114.3±3.7 vs. 85.5±5.2 mmHg).

Both groups showed similar degree of proteinuria, with no significant difference in urinary protein/creatinine ratio at any time point (Figure S4a). With hydralazine treatment, glomerulosclerosis was not attenuated, with sclerosis index, on average 0.11±0.06, which was not statistically different from that in the control group, 0.37±0.11 (Figure S4b). Similarly, there was no significant difference between the two groups in nephrin index (7.75±0.13 vs. 7.03±0.20)(Figure S4c).
Figure S1

Generation of conditional Agtr1a targeted mice.
(a) Wild-type Agtr1a. The entire coding region is included within exon 3.
(b) Construction of the targeting vector. The targeting vector contains 13 kb of intron 2, exon 3, 2.8 kb of 3’ flanking region. At the Bgl II site in intron 2, a loxP sequence (shown by a triangle) was inserted. At the Pst I site in 3’ flanking region, a neomycin resistant gene cassette (pgk-neo) flanked by two loxP sites was inserted. At the 3’ end of the targeting vector, an expression cassette for herpes simplex virus thymidine kinase (pgk-th) was attached for negative selection.
(c) Structure of mutant Agtr1a (Agtr1aneo) obtained by homologous recombination with (b).
(d) Structure of mutant Agtr1a (Agtr1aloxp) in the ES cell clone used for microinjection. The bulky pgk-neo of Agtr1aneo was removed by transient transfection with pCre-Pac plasmid.
(e) Structure of null Agtr1a allele (Agtr1a-) generated by Cre-mediated recombination. In Agtr1aloxp/loxP/Nephrin-Cre mice, only podocytes carry this allele, while other types of cells have Agtr1aloxp.
(f) Southern analysis for ES cell screening. Genomic DNA digested with BamH I was hybridized with the probe shown in (a). 8 kb bands represent wild-type Agtr1a allele and 5 kb bands (*) represent Agtr1aneo allele.
Figure S2

Effect of ARB treatment starting after LMB2 injection on NEP25 mice.

NEP25 mice were injected with LMB2 (0.625 ng/g BW). Mice with ARB treatment starting 12 hours after LMB2 injection (closed columns) showed significantly attenuated urinary albumin/creatinine ratio (a), glomerulosclerosis (b) and downregulation of nephrin (c) when examined 4 weeks after the injection, compared to those in control mice without ARB (open columns). * p<0.05.

In this experiment, Agtr1a<sup>loxP/loxP/Cre(-)/NEP25</sup> mice were used. These and the mice used in the experiment shown in Figure 2 were from the same colony. Without LMB2, both strains of mice showed no sclerosis or podocyte damage with sclerosis index 0, and nephrin index 8.

* p<0.05.
Figure S3

*Effect of captopril treatment starting after LMB2 injection on NEP25 mice.*

NEP25 mice were injected with LMB2 (0.625 ng/g BW). Mice with captopril treatment starting 12 hours after LMB2 injection (closed columns) showed significantly attenuated urinary albumin/creatinine ratio 1-4 weeks after the injection (a), glomerulosclerosis (b) and downregulation of nephrin (c) when compared to those in control mice without captopril (open columns). Without LMB2, both strains of mice showed no sclerosis or podocyte damage with sclerosis index 0, and nephrin index 8.

* p<0.05.
Figure S4

Effect of hydralazine on NEP25 mice.

Hydralazine treatment starting 5 days before LMB2 injection (0.625 ng/g BW) exerted no impact on urinary total protein/creatinine ratio (a), glomerulosclerosis (b) or downregulation of nephrin (c).