Angiotensin Receptor Blocker Protection Against Podocyte-Induced Sclerosis Is Podocyte Angiotensin II Type 1 Receptor-Independent

Taiji Matsusaka, Takako Asano, Fumio Niimura, Masaru Kinomura, Akihiro Shimizu, Ayumi Shintani, Ira Pastan, Agnes B. Fogo, Iekuni Ichikawa

Abstract—In the present study, we tested the hypothesis that the renoprotective effect of an angiotensin receptor blocker depends on the angiotensin II type 1 (AT₁) receptor on podocytes. For this purpose, we generated podocyte-specific knockout mice for the AT₁ gene (Agrp1a) and crossed with NEP25, in which selective podocyte injury can be induced by immunotoxin, anti-Tac(Fv)-PE38. Four weeks after the addition of anti-Tac(Fv)-PE38, urinary albumin:creatinine ratio was not attenuated in Agrp1a knockout/NEP25 mice (n=18) compared with that in control NEP25 mice (n=13; 8.08±2.41 in knockout versus 4.84±0.73 in control). Both strains of mice showed similar degrees of sclerosis (0.66±0.17 versus 0.82±0.27 on a 0 to 4 scale) and downregulation of nephrin (5.78±0.45 versus 5.65±0.58 on a 0 to 8 scale). In contrast, AT₁ antagonist or an angiotensin I–converting enzyme inhibitor, but not hydralazine, remarkably attenuated proteinuria and sclerosis in NEP25 mice. Moreover, continuous angiotensin II infusion induced microalbuminuria similarly in both Agrp1a knockout and wild-type mice. Thus, angiotensin inhibition can protect podocytes and prevent the development of glomerulosclerosis independent of podocyte AT₁. Possible mechanisms include inhibitory effects on AT₁ of other cells or through mechanisms independent of AT₁. Our study further demonstrates that measures that directly affect only nonpodocyte cells can have beneficial effects even when sclerosis is triggered by podocyte-specific injury. (Hypertension. 2010;55:967-973.)

Key Words: podocyte ■ glomerulosclerosis ■ chronic renal failure ■ AT₁ antagonist ■ knockout mice ■ proteinuria

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 clinically established therapeutic measure for slowing the progression of chronic kidney diseases. ACE inhibitors and ARBs have been shown to attenuate podocyte damage, proteinuria, and development of glomerulosclerosis in a variety of animal models, including, among others, the subtotal nephrectomy model and diabetic nephropathy models.

Continuous infusion of Ang II in normal rats increased 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. 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 Podocytes in isolated glomeruli express functional AT₁, and Ang II depolarizes and increases intracellular Ca²⁺.

In cultured podocytes, Ang II, via AT₁, increases α3(IV) collagen and...
vascular endothelial growth factor decreases nephrin, heparan sulfate proteoglycans, and α-actinin; 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−deficient mice. Unlike humans, mice have 2 AT1 receptor genes, Agtr1a and Agtr1b, each encoding AT1A and AT1B receptor subtype, respectively. In the mouse kidney, >99% of AT1 mRNA is derived from Agtr1a, and Agtr1a inactivation does not lead to activation of Agtr1b. We, therefore, used podocyte-specific Agtr1a 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 Agtr1a Null-Mutant Mice**

Detailed methods for generation of Agtr1aloxp mice (C57BL/6 background) are described in the online Data Supplement (please see hyper.ahajournals.org). Nephrin-Cre mice were reported previously. In the present study, line 10 of Nephrin-Cre mice was used. They were backcrossed with the C57BL/6 strain >3 times and used for mating with Agtr1aloxp mice. To induce podocyte-specific injury, mice carrying Agtr1aloxp and Nephrin-Cre were further mated with NEP25 mice on the C57BL/6 genetic background.

**Determination of Genotype of Podocytes**

From mice carrying the Agtr1aloxp/Cre(+)TRE-SV40T/podocin-rtTA/ROSA26loxP, or Agtr1aloxp+/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 5 days. Sprouting cells were sparsely replated and cultured until they formed colonies. Cells were then fixed in 2% glutaraldehyde/PBS for 10 minutes and stained for la2c in a staining solution (2 mmol/L of MgCl2, 0.02% Nondet-P40, 0.01% Na deoxycholic acid, 5 mmol/L of K,Fe(CN)6, 5 mmol/L of K3[Fe(CN)6], and 1 mg/mL of 5-bromo-4-chloro-3-indolyl β-D-galactoside in PBS [pH 7.4]) at 37°C for 4 hours. After washing with PBS, isolated colonies were lysed in a solution (10 mmol/L of Tris-HCl, 1 mmol/L of EDTA, 1% Tween 20, and 0.4 mg/mL of protease K) at 55°C for 12 hours. The lysate containing genomic DNA was harvested, heated to inactivate proteinase K, and used as a template of PCR.

The following 3 primers were used: AT5, AT6, and AT7. Amplification of AGTR1A and AGTR1B mRNA was performed using the primer pair AT6 and AT7 (Figure 1). Embryonic stem (ES) cell DNA carrying Agtr1a+/Cre(+)TRE-SV40T/podocin-rtTA/ROSA26loxP. Both show the Agtr1a+/− genotype. Other 6 clones (not shown) similarly showed the Agtr1a+/− genotype. Lanes 8 to 12, Lac-Z-tagged podocyte clones from mice carrying Agtr1aloxp/loxP/Cre(+)TRE-SV40T/podocin-rtTA/ROSA26loxP. All show the Agtr1a+/− genotype. Thirteen (87%) of 15 clones examined showed the Agtr1a+/− genotype.

**Figure 1.** Verification of Agtr1a disruption in the podocytes of Agtr1aloxp/loxP/Crl+ mice. A, PCR for the genotyping of Agtr1a. In wild-type Agtr1a, the primer pair AT6 and AT7 generated a 300-bp band. The primer pair AT5 and AT7 did not amplify DNA. In Agtr1aloxp, the primer pair AT6 and AT7 generated a 366-bp band, whereas the primer pair AT5 and AT7 did not amplify DNA. In Agtr1a−/−, the primer pair AT5 and AT7 generated a 213-bp band. The portion recognized by AT6 is removed from the Agtr1a−/− genome. B, Representative PCR result. Lane 1, DNA size markers. Lane 2, ES cell DNA carrying Agtr1a+/+. Lane 3, ES cell DNA carrying Agtr1a+/−. Lane 4, Tail DNA carrying Agtr1a−/− (wild type). Lane 5, Tail DNA carrying Agtr1aloxp/loxP. Lanes 6 and 7, Lac-Z-tagged podocyte clones from mice carrying Agtr1aloxp/loxP/Crl+TRE-SV40T/podocin-rtTA/ROSA26loxP. Both show the Agtr1a+/− genotype. Other 6 clones (not shown) similarly showed the Agtr1a+/− genotype. Lanes 8 to 12, Lac-Z-tagged podocyte clones from mice carrying Agtr1aloxp/loxP/Crl+TRE-SV40T/podocin-rtTA/ROSA26loxP. All show the Agtr1a+/− genotype.
PCR was carried out using primers GCATCATTTTGTGGTGGG and GAAGAAAAAGCACATGCC agar, 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 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

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(+/NEP25) and 10 female and 8 male mice carrying Agtr1aloxP/loxP/Cre(+/NEP25) (3 to 7 months of age) were used. Twenty-four–hour urine was collected before and 7, 14, 21, and 28 days after LMB2 injection. They were euthanized 28 days after LMB2 injection.

To study the effect of losartan, an AT1 receptor blocker, 8 female NEP25 mice with a C57BL/6 genetic background (5 months of age) were treated with losartan (0.5 g/L, in drinking water, ~25 μg/g of BW) from 5 days before LMB2 injection until the end of the experiment. Nine age- and sex-matched NEP25 mice were used as controls. In both groups of mice, 0.625 ng/g of BW of LMB2 were intravenously injected under diethyl ether anesthesia. Conscious systolic blood pressure was measured by tail-cuff method using MK-2000 (Mamarachi Kikai) 2 days before LMB2 injection. Systolic blood pressure measured by MK-2000 is reported to be well available to us, and we, therefore, cultured podocytes and then cloned and determined the Agtr1a genotype by PCR. For this purpose, Agtr1aloxP/loxP/Cre(+) mice were 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, we next tested whether recombination occurs in similar efficiency in podocytes of Agtr1aloxP/loxP/Nephrin-Cre (Agtr1aloxP/loxP/Cre(+) mice). Reliable antitoxin AT1 antibodies suitable for immunohistochemical study were not available to us, and we, therefore, cultured podocytes and then cloned and determined the Agtr1a genotype by PCR. For this purpose, Agtr1aloxP/loxP/Cre(+) mice were mated with TRE-SV40T/podocin-rTA 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 the ROSA26loxP line.

Confirmation of Agtr1a Disruption in Podocytes of Agtr1aloxP/loxP/Cre(+) Mice

The Agtr1aloxP/loxP 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, we next tested whether recombination occurs in similar efficiency in podocytes of Agtr1aloxP/loxP/Nephrin-Cre (Agtr1aloxP/loxP/Cre(+) mice). Reliable antitoxin AT1 antibodies suitable for immunohistochemical study were not available to us, and we, therefore, cultured podocytes and then cloned and determined the Agtr1a genotype by PCR. For this purpose, Agtr1aloxP/loxP/Cre(+) mice were mated with TRE-SV40T/podocin-rTA 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 the ROSA26loxP line.

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.

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

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

Podocyte-selective AT	extsubscript{1} knockout mice, Agtr1a	extsuperscript{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	extsuperscript{loxP/loxP}/Cre(+) mice was not different from that in control Agtr1a	extsuperscript{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	extsuperscript{loxP/loxP}/Cre(+) mice was not different from that in Agtr1a	extsuperscript{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	extsuperscript{loxP/loxP}/Cre(+) mice were normal over a range from age 1 to 12 months (data not shown).

To determine the relative amount of AT	extsubscript{1}A and AT	extsubscript{1}B mRNA, RT-PCR was performed in glomerular RNA from Agtr1a	extsuperscript{loxP/loxP}/Cre(+) and Agtr1a	extsuperscript{loxP/loxP}/Cre(−) mice using primers common to AT	extsubscript{1}A and AT	extsubscript{1}B sequences following digestion with EcoRI, which is specific to AT	extsubscript{1}A. AT	extsubscript{1}B mRNA was undetectable in glomeruli of either type of mice with this method. Real-time RT-PCR revealed that AT	extsubscript{1}B mRNA was detectable in the glomerulus, but the quantity was <0.5% (0.0% to 0.5%; n=4) of that of AT	extsubscript{1}A in either mice. Without LMB2, both types of mice showed similar degrees of glomerulosclerosis (B and C) and a similar degree of downregulation of nephrin (E and F, arrows). A through C, periodic acid Schiff, ×200; D through F, antinephrin, ×400.

**Effect of Podocyte-Specific Inactivation of AT	extsubscript{1} on Progression of Glomerulosclerosis**

To examine whether podocyte-specific deletion of AT	extsubscript{1} receptor can slow the progression of glomerulosclerosis, we mated Agtr1a	extsuperscript{loxP/loxP}/Cre(+) mice with the NEP25 line, generating Agtr1a	extsuperscript{loxP/loxP}/Cre(+)NEP25 and Agtr1a	extsuperscript{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	extsuperscript{loxP/loxP}/

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Figure 2. Effect of podocyte-specific AT1 deletion on glomerular injury. Agtr1a	extsuperscript{loxP/loxP}/Cre(−)/NEP25 (○) and Agtr1a	extsuperscript{loxP/loxP}/Cre(+) mice were injected with LMB2 (0.625 ng/g of BW). There was no significant difference in urinary albumin:creatinine ratio (ACR; A), sclerosis index (B), or nephrin index (C) 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.

Figure 3. Representative pictures of Agtr1a	extsuperscript{loxP/loxP}/Cre(−)/NEP25 and Agtr1a	extsuperscript{loxP/loxP}/Cre(+)NEP25 mice. Without LMB2, Cre (+) mice showed normal glomerular morphology (A) and a normal nephrin staining pattern (D). After LMB2 injection, both Cre (−) and Cre (+) mice showed similar degrees of glomerulosclerosis (B and C) and a similar degree of downregulation of nephrin (E and F, arrows). A through C, periodic acid Schiff, ×200; D through F, antinephrin, ×400.
Cre(+/−)/NEP25 mice had a sclerosis index of 0.65±0.16, which was not statistically different from that of Agtr1aloxP/loxP/Cr(−)/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/Cr(+/−)NEP25 mice was, on average, 5.78±0.45, which was not statistically different from that of Agtr1aloxP/loxP/Cr(−)/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, 0 to 8 scale; Figures 4C and 5). Similar protection was observed in Agtr1aloxP/loxP/Cr(+/−)NEP25 mice treated with ARB (Figure 6). Agtr1aloxP/loxP/Cr(−)/NEP25 mice treated with ARB starting after LMB2 injection (Figure 6 versus 101

**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/Cr(−) and Agtr1aloxP/loxP/Cr(+). 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
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 receptor(s).

As discussed earlier, podocytes in vitro, as well as in vivo, have been shown to express functional AT1.25,26 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.27 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 (Agtr1a<sup>loxP/loxP</sup>/Cre<sup>−/−</sup>/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.31–34 Recently, Crowley et al35 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 AT1 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-AI1A 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.

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.34–36

**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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**ARB Protection Against Podocyte-Induced Sclerosis is Podocyte AT1-Independent.**

Short title: Podocyte AT1 and Glomerulosclerosis

Taiji Matsusaka\(^1, 4, 5\), Takako Asano\(^6\), Fumio Niimura\(^6\), Masaru Kinomura\(^5\), Akihiko Shimizu\(^10\), Ayumi Shintani\(^11\), Ira Pastan\(^8\), Agnes B. Fogo\(^1, 2, 3\), Iekuni Ichikawa\(^1, 3, 7\)

Departments of \(^1\)Pediatrics, \(^2\)Pathology, \(^3\)Medicine, and \(^11\)Biostatistics, Vanderbilt University Medical Center, Nashville, TN, USA

\(^4\)Institute of Medical Science, \(^5\)Departments of Internal Medicine, \(^6\)Pediatrics and \(^7\)Bioethics, Tokai University School of Medicine, Isehara, Kanagawa, Japan

\(^8\)Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland, USA

\(^9\)Department of Pediatrics, National Defense Medical College, Tokorozawa, Saitama, Japan

\(^10\)Division of Kidney and Hypertension, Department of Internal Medicine, Jikei University School of Medicine, Tokyo, Japan

Corresponding Author:

Taiji Matsusaka

Department of Internal Medicine

Tokai University School of Medicine

143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan

Email: taijim@is.icc.u-tokai.ac.jp

FAX: 81-463-90-1611

Phone: 81-463-93-1121
Methods

Generation of Agtr1a<sup>loxP</sup> 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 BglII site of intron 2, upstream of the coding exon, one loxP site was inserted. At the PstI 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<sup>loxP</sup>. One of the targeted ES cells was injected into C57BL/6 blastocysts. One of the 10 chimeric mice obtained showed germline transmission of Agtr1a<sup>loxP</sup> 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<sup>loxP</sup>. Heterozygous (Agtr1a<sup>loxP/+</sup>) 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<sup>loxP</sup>, in which two loxP sites were inserted before and after the coding exon of Agtr1a (Figure S1). Northern blotting analysis
revealed that $Agtr1a^{lackP/loxP}$ and $Agtr1a^{lackP/+}$ 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 $Agtr1a^{lackP/loxP}$ and $Agtr1a^{lackP/+}$ mice, respectively, which was not different statistically from that in wild-type littermates (100±12 mmHg). $Agtr1a^{lackP/loxP}$ and $Agtr1a^{lackP/+}$ 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 $Agtr1a^{lackP/loxP}$ 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 ROSA26$^{loxP}$, a tester strain. 100% of podocytes were lacZ positive in Nephrin-Cre/ROSA26$^{loxP}$. We next tested whether recombination occurs in similar efficiency in podocytes of $Agtr1a^{lackP/loxP}$/Nephrin-Cre ($Agtr1a^{lackP/loxP}$/Cre(+)) 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, $Agtr1a^{lackP/loxP}$/Cre(+) 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 ROSA26$^{loxP}$ line.

Glomeruli obtained from $Agtr1a^{lackP/loxP}$/Cre(+)/TRE-SV40T/podocin-rtTA/ROSA26$^{loxP}$ 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 ($Agtr1a$)(Figure S2). Two lacZ positive colonies showed both $Agtr1a^{lackP}$ and $Agtr1a$, indicating that they are heterozygote ($Agtr1a^{lackP}$). LacZ-negative cobblestone-like cells often grew even without doxycycline. PCR analysis revealed that all three such colonies examined had $Agtr1a^{lackP/loxP}$ genotype. Similar analysis in eight lacZ positive colonies from $Agtr1a^{lackP/+}$/Cre(+)/TRE-SV40T/podocin-rtTA/ROSA26$^{loxP}$ showed that all lacZ-positive clones had $Agtr1a^{+/+}$ genotype. These confirmed that Cre-mediated recombination of $Agtr1a^{lackP}$ occurs efficiently in podocytes and most podocytes in $Agtr1a^{lackP/loxP}$/Cre(+) 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±076 (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-tk) 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 BamHI 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</sup>/Cre<sup>-</sup>/NEP25 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).