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Podocyte injury enhances filtration of liver-derived angiotensinogen and renal angiotensin II generation

Taiji Matsusaka[1,2], Fumio Niimura[3], Ira Pastan[4], Ayumi Shintani[5,6], Akira Nishiyama[7] and Iekuni Ichikawa[6,8,9]

[1]Department of Internal Medicine, Tokai University School of Medicine, Isehara, Japan; [2]Institute of Medical Science, Tokai University School of Medicine, Isehara, Japan; [3]Department of Pediatrics, Tokai University School of Medicine, Isehara, Japan; [4]Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland, USA; [5]Department of Biostatistics, Vanderbilt University Medical Center, Nashville, Tennessee, USA; [6]Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee, USA; [7]Department of Pharmacology, Research Equipment Center, and Second Department of Internal Medicine, Kagawa University School of Medicine, Kagawa, Japan; [8]Tokai University School of Medicine, Isehara, Japan and [9]Department of Pediatrics, Vanderbilt University Medical Center, Nashville, Tennessee, USA

Intrarenal angiotensin II is increased in kidney diseases independently of plasma angiotensin II and is thought to promote progressive deterioration of renal architecture. Here we investigated the mechanism of enhanced renal angiotensin II generation in kidney glomerular diseases. For this, kidney- or liver-specific angiotensinogen gene (*Agt*) knockout was superimposed on the mouse model of inducible podocyte injury (NEP25). Seven days after induction of podocyte injury, renal angiotensin II was increased ninefold in NEP25 mice with intact *Agt*, accompanied by increases in urinary albumin and angiotensinogen excretion, renal angiotensinogen protein, and its mRNA. Kidney *Agt* knockout attenuated renal *Agt* mRNA but not renal angiotensin II, renal, or urinary angiotensinogen protein. In contrast, liver *Agt* knockout markedly reduced renal angiotensin II to 18.7% of that of control NEP25 mice, renal and urinary angiotensinogen protein, but not renal *Agt* mRNA. Renal angiotensin II had no relationship with renal *Agt* mRNA, or with renal renin mRNA, which was elevated in liver *Agt* knockouts. Kidney and liver dual *Agt* knockout mice showed phenotypes comparable to those of liver *Agt* knockout mice. Thus, increased renal angiotensin II generation upon severe podocyte injury is attributed to increased filtered angiotensinogen of liver origin resulting from loss of macromolecular barrier function of the glomerular capillary wall that occurs upon severe podocyte injury.

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Correspondence: *Taiji Matsusaka, Department of Internal Medicine, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan. E-mail: taijim@is.icc.u-tokai.ac.jp*

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In many forms of kidney disease, pharmacological intervention of angiotensin II (AII) has salutary effects, although plasma AII is generally not elevated. In normal condition, intrarenal AII is above its level in the plasma, and increases further in chronic kidney diseases.[1–9] These findings have collectively formed the prevailing notion that the intrarenal AII is regulated differently from plasma AII, thereby exerting detrimental effects on the renal structure. Among the several mechanisms proposed[7–19] for the increased AII, transcriptional activation of the renal angiotensinogen (*Agt*) gene is thought to play a central role in view of readily demonstrable expression of *Agt* mRNA in the kidney.

Agt protein mainly exists in proximal convoluted tubules (S1 and S2 segments). On the other hand, S3 segment contains more *Agt* mRNA than S1 and S2,[20,21] although earlier reverse transcriptase–PCR study by Terada *et al.*[22] showed that both proximal convoluted and straight tubules express *Agt* mRNA. Nevertheless, concurrent increases in *Agt* mRNA and protein seen in renal tissue homogenates have been taken to indicate that transcriptional activation of the renal *Agt* gene causes an increase in renal AII.

In this regard, Pohl *et al.*[21] elegantly demonstrated that Agt is a ligand of megalin and that the renal Agt protein detectable by immunostaining is taken up from glomerular filtrate by megalin-dependent endocytosis and its localization is distinct from that of *Agt* mRNA.

We recently demonstrated that kidney (proximal straight tubule)–specific *Agt* knockout (KO) mice have markedly decreased renal *Agt* mRNA, but renal Agt protein and AII content are unaffected.[23] In contrast, KO of the liver *Agt* gene near completely abolishes renal Agt protein and markedly decreases AII contents. Thus, clearly, the liver-derived Agt is the major source of renal AII in basal condition. This observation led us to speculate that the functional integrity of glomerular capillary wall as a molecular barrier may determine the renal AII synthesis in progressive glomerular diseases. To test this idea, we analyzed NEP25 mice in which

injury can be induced in a podocyte-specific manner by injection of immunotoxin, LMB2.[24] After induction of podocyte injury, renal Agt protein and AII increased along with an increase in urinary Agt excretion. Moreover, analysis in NEP25 mice carrying mosaic tubular megalin KO revealed that tubular distribution of Agt protein in proteinuric state is also completely dependent on megalin. These collectively suggested that an increase in the leakage of plasma Agt into tubular lumen is the major mechanism of increased renal AII in proteinuric glomerular diseases.

Earlier studies by others, however, reported that in various disease models,[11,25–28] the *Agt* transcription in the proximal tubule is enhanced, raising the possibility that renal *Agt* mRNA may contribute to renal AII synthesis in kidney diseases.[29] This study aims to determine the contribution of the kidney- versus liver-derived Agt to the renal AII generation in glomerular diseases with podocyte injury.

RESULTS

Podocyte injury results in massive leakage and renal incorporation of liver-originated Agt protein

We generated NEP25 mice carrying four types of *Agt* genotypes: control intact *Agt* ($Agt^{loxP/loxP}$), kidney *Agt* KO (*KAP-Cre:*$Agt^{loxP/loxP}$), liver *Agt* KO (*albumin-Cre:*$Agt^{loxP/loxP}$), and dual *Agt* KO (*KAP-Cre:albumin-Cre:*$Agt^{loxP/loxP}$). In kidney *Agt* KO mice, the *Agt* gene in proximal tubules is mostly disrupted, and renal *Agt* mRNA level is decreased to 14% of that in control or wild-type mice. In liver *Agt* KO mice, the *Agt* gene in the liver is near completely knocked out.[23] The four types of mice were injected with LMB2 (0, 1.25, or 2.5 ng per g body weight (BW)) to induce podocyte injury.

At 7 days after 1.25 or 2.5 ng per g BW of LMB2, all types of mice developed massive proteinuria, with urinary albumin/creatinine (Cr) ratio >60 mg/mg (Figure 1). Analysis in separate mice indicated that blood pressure is not affected by 2.5 ng per g BW of LMB2 in control, kidney, and liver *Agt* KO mice (Supplementary Table S1 online). Cystatin C did not significantly change (Supplementary Table S2 online).

The mice injected with LMB2 did not develop glomerulosclerosis or interstitial fibrosis at this time point (Supplementary Figure S1 online). Nephrin staining was diminished, depending on the dose of LMB2 (Figure 2a and Supplementary Figure S2 online). Liver *Agt* KO and dual *Agt* KO mice showed significantly more preserved nephrin after 1.25 ng per g BW of LMB2 than control *Agt* mice given the same does of LMB2. However, after 2.5 ng per g BW of LMB2, mice of all genotypes showed comparably severe nephrin loss (Figure 2a). Control and kidney *Agt* KO mice similarly showed early sign of tubulointerstitial damage, including tubule dilatation, tubule apoptosis, and proliferation (Ki67) of tubular and interstitial cells (Figure 2b–e). There was a tendency that liver or dual *Agt* KO mice develop milder tubule apoptosis and Ki67 after 1.25 ng per g BW of LMB2, but more severe tubule dilatation and interstitial Ki67 after 2.5 ng per g BW of LMB2.

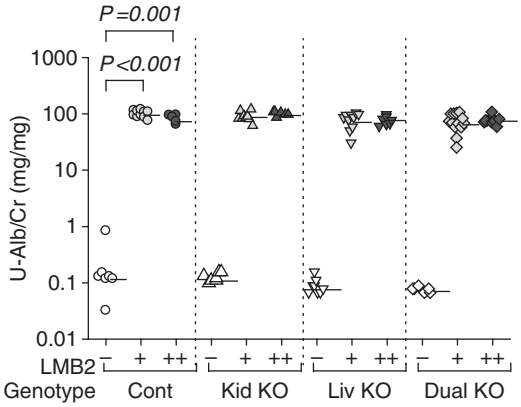

Figure 1 | Urinary albumin/creatinine ratio (U-Alb/Cr). NEP25 mice with control *Agt* genotype (Cont), kidney *Agt* knockout (Kid KO), liver *Agt* KO (Liv KO), and dual *Agt* KO (Dual KO) were injected with 1.25 or 2.5 ng per g body weight (BW) of LMB2 (depicted by '+' and '++', respectively) to induce podocyte injury or vehicle (–) and analyzed 7 days later. All types of mice developed massive proteinuria after LMB2 injection. Horizontal bars represent geographical means. The statistical method is described in detail in the legend of Figure 3. For comparison among genotypes at each LMB2 dose, data of Figures 1, 3, 5, and 7a are presented separately for each LMB2 dose in Supplementary Figure S3 online. Representing numerical values are shown in Supplementary Table S3 online.

In control *Agt* mice, LMB2 markedly and dose-dependently increased renal Agt protein (Figure 3). Kidney *Agt* KO showed similar level of Agt protein at the basal condition and similarly increased Agt after injection of LMB2. In contrast, liver *Agt* KO markedly reduced renal Agt protein in both podocyte-intact and podocyte-injured conditions. Dual *Agt* KO mice showed similar patterns to those of liver *Agt* KO mice.

Immunostaining for Agt paralleled to the result of western analysis. Thus, after injection of LMB2, control and kidney *Agt* KO mice showed similarly intense granular staining in proximal tubule cells (Figure 4). Along the S3 segment, where *Agt* mRNA is intensely expressed, kidney *Agt* KO did not attenuate Agt staining to any appreciable extent. In contrast, liver and dual *Agt* KO mice showed almost no staining after 1.25 ng per g BW of LMB2. After 2.5 ng per g BW of LMB2, although focal Agt staining was discernible along with proteinaceous casts, it was far less than that in control or kidney *Agt* KO mice.

Similar trend was observed in urinary Agt excretion but in a more dramatic manner. Thus, urinary Agt/Cr ratio increased $\sim$300-fold after 1.25 ng per g BW of LMB2, and 1300-fold after 2.5 ng per g BW of LMB2 in control mice (Figure 5). Kidney *Agt* KO mice showed similar patterns to those in control mice. In contrast, Agt/Cr ratio in liver *Agt* KO mice after 1.25 and 2.5 ng per g BW of LMB2 remained at levels of only 5% and 3%, respectively, of those in control mice injected with the same dose of LMB2. Dual *Agt* KO mice showed similarly low urinary Agt. Within control and kidney