Hepatocyte Growth Factor Attenuates Transforming Growth Factor-β-Angiotensin II Crosstalk Through Inhibition of the PTEN/Akt Pathway

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Abstract—Both angiotensin II (Ang II) and transforming growth factor (TGF)-β1 are thought to be involved in the progression of chronic kidney disease. In contrast, hepatocyte growth factor (HGF) counteracts the actions of Ang II and TGF-β1. Therefore, in this study, we investigated the molecular mechanisms of how HGF antagonizes the Ang II-TGF-β axis in renal cells. In cultured human mesangial cells, TGF-β1 increased angiotensin type 1 receptor (AT1R) mRNA, mainly dependent on the Akt/phosphatidylinositol 3-kinase signaling pathway. Furthermore, TGF-β1 decreased the expression and phosphatase activity of phosphatase and tensin homolog, deleted on chromosome 10 (PTEN), a negative regulator of the phosphatidylinositol 3-kinase/Akt pathway. These data revealed positive feedback of the Ang II-TGF-β pathway, because Ang II increased TGF-β expression. In contrast, HGF significantly attenuated the increase in AT1R gene expression, and inhibited the decrease in PTEN induced by TGF-β1. Of importance, a PTEN-specific inhibitor significantly attenuated the reduction in TGF-β1–induced AT1R expression by HGF. These data suggest that HGF attenuated TGF-β1–induced AT1R expression through the PTEN/Akt pathway. To investigate this hypothesis, we performed in vivo experiments in mice with increased circulating levels of HGF produced by transgenically expressing HGF under control of a cardiac-specific transgene (HGF-Tg). In HGF-Tg mice, renal injury and fibrosis were significantly decreased, associated with reduction in AT1R expression and increase in PTEN after Ang II infusion, as compared with control mice. Moreover, these renal protective effects were abrogated by a neutralizing antibody against HGF. Thus, the present study demonstrated that HGF counteracts the vicious cycle of Ang II–TGF-β1–AT1R, mediating the inhibition of PTEN. (Hypertension. 2011;58:190-196.) • Online Data Supplement

Key Words: fibrosis ■ TGF-β HGF ■ CKD ■ PTEN

Maladaptive activation of the renin-angiotensin system (RAS) has been shown to play a pivotal role in the pathogenesis of chronic kidney disease (CKD). Angiotensin II (Ang II), the major bioactive peptide of RAS, is a potent systemic vasoconstrictor and modulator of the renal circulation. Ang II has important nonhemodynamic effects that have been implicated in the pathogenesis of CKD by promoting mesangial cell hypertrophy and proliferation, increasing extracellular matrix synthesis1 and promoting inflammation.2 The biological responses to Ang II are mediated by 2 plasma membrane receptors, Ang II type 1 receptor (AT1R) and Ang II type 2 receptor.3 Most of the known pathophysiological effects of Ang II are mediated by AT1R. In both clinical and experimental studies, angiotensin-converting enzyme (ACE) inhibitors and Ang II type 1 receptor blockers (ARBs) clearly revealed renoprotective effects. In contrast, transforming growth factor-β (TGF-β) is also well known to be a key mediator in the progression of CKD. TGF-β1 has been linked mainly to fibrinogenesis in some animal models. One of the striking effects of TGF-β1 is stimulation of AT1R expression, whereas the proatherosclerotic action of Ang II is mediated, at least in part, by TGF-β1. Thus, the positive feedback loop of Ang II and TGF-β1 might result in amplification of their profibrotic effects in renal cells.

In contrast, hepatocyte growth factor (HGF) is a pleiotropic factor that plays an essential role in the regulation of cell proliferation, survival, and differentiation in a variety of organs. Specifically, HGF prevented renal interstitial fibrosis in several animal models of CKD. Recent studies suggest that HGF counteracts the action of TGF-β1 in different types of kidney cells, resulting in inhibition of TGF-β–mediated myofibroblastic activation of glomerular mesangial cells and interstitial fibroblasts and blockade of tubular epithelial-to-mesenchymal transition. In general, it is believed that HGF...
antagonizes the profibrotic actions of TGF-β1 by intercepting Smad signal transduction in different kidney cells and the injured kidney. In addition, our group demonstrated recently that HGF promoted apoptosis of myofibroblasts, an important source of TGF-β1. However, the molecular mechanisms of inhibition of the Ang II-TGFβ pathway by HGF have not been fully clarified. Thus, in this study, we investigated how HGF counteracts the positive feedback loop between Ang II and TGF-β1 that results in amplification of their profibrotic effects in the kidney.

### Materials and Methods

Please see the online Data Supplement (http://hyper.ahajournals.org) for information on the primary antibodies and reagents used in the study.

### In Vitro Experiments

Human mesangial cells were cultured in mesangial cell basic culture medium plus 5% FBS. The cells were seeded to 70% to 80% confluence in complete medium and then changed to serum-free medium. Recombinant human TGF-β1 was added to the cultures at a final concentration of 1 ng/mL.

### Western Blot Analysis and Quantitative Real-Time PCR

Preparation of protein extract and Western blotting were performed as described previously. Total RNA was isolated as described previously. In each experiment, mouse β-actin RNA was amplified as a reference standard.

### In Vivo Experiments

We generated mice (C57BL6 background) with cardiac-specific (α-major histocompatibility complex–driven) overexpression of PTEN in Inhibition of AT1R

### Statistical Analysis

Values are expressed as mean±SE. ANOVA and t test followed by Bonferroni adjustment for multiple comparisons were used for comparisons of >2 groups. A P value <0.05 was considered to indicate significance of difference of mean values.

### Results

#### Role of Phosphatase and Tensin Homolog, Deleted on Chromosome 10 (PTEN) in Inhibition of AT1,R Expression by HGF

To elucidate the molecular mechanisms of renal injury by Ang II, we first tested the effect of TGF-β1 on AT1,R mRNA expression in human mesangial cells, because our previous study demonstrated that Ang II stimulated TGF-β1 mRNA expression in human mesangial cells. As shown in Figure S1 (available in the online Data Supplement), TGF-β1 significantly inhibited the increase in AT1,R mRNA expression by Ang II (Figure 1A). Of importance, pretreatment with HGF significantly attenuated the increase in AT1,R mRNA expression induced by TGF-β1 with pharmacological inhibitors of signaling pathways. Expression of AT1,R was determined by quantitative PCR using β-actin as an internal control.

### Figure 1. Effect of hepatocyte growth factor (HGF) on angiotensin (Ang) (Ang) II type 1 receptor (AT1,R) mRNA expression in vitro. A, AT1,R mRNA expression induced by transforming growth factor (TGF)-β1 with Ang II and HGF. Expression of AT1,R was determined by quantitative PCR using β-actin as an internal control. Data represent mean±SEM. *P<0.05 vs control, †P<0.05 vs TGF-β1. B, AT1,R mRNA expression induced by TGF-β1 with pharmacological inhibitors of signaling pathways. Expression of AT1,R was determined by quantitative PCR using β-actin as an internal control. Data represent mean±SEM. *P<0.05 vs non-treated, †P<0.05 vs TGF-β1. LY indicates LY294002, phosphatidylinositol 3-kinase (PI3K) inhibitor (1×10⁻⁵ M); PD, PD98059, extracellular signal–regulated kinase (ERK) 1/2 inhibitor (2×10⁻⁵ M); SB, SB203580, p38 inhibitor (2×10⁻⁵ M); SP, SP600125, c-Jun N-terminal kinase (JNK) inhibitor (1×10⁻⁵ M).
extracellular signal–regulated kinase 1/2 (by PD98059) and p38 (by SB203580) did not affect it, indicating that the increase in AT_{1}R expression induced by TGF-β1 might be mainly through the PI3K-Akt signaling pathway.

Therefore, we focused on the HGF-PI3K/Akt pathway. TGF-β1 significantly increased the phosphorylation of Akt, which was maximal at 15 minutes (data not shown). However, pretreatment with HGF significantly attenuated Akt phosphorylation stimulated by TGF-β1 (Figure 2A and 2B; *P<0.01). Because PI3K action is regulated by the tumor suppressor protein, phosphatase and tensin homolog, deleted on chromosome 10 (PTEN), by dephosphorylation of phosphatidylinositol (PI) 3,4,5-trisphosphate,10,11 we next focused on the role of PTEN. Interestingly, as shown in Figure S2A, TGF-β1 significantly decreased the expression of PTEN in a time-dependent manner, whereas HGF markedly attenuated the decrease in PTEN expression induced by TGF-β1 (Figure S2B; †P<0.01). Of importance, a PTEN inhibitor (bpV(OH)pic) significantly inhibited the attenuation of TGF-β1–induced Akt activation by HGF (Figure 2A and 2B; †P<0.01). To elucidate the role of PTEN in the increase in AT_{1}R expression induced by TGF-β1, we further studied the effects of a PTEN inhibitor on AT_{1}R expression. Of importance, as shown in Figure 2C, the addition of a PTEN inhibitor significantly prevented the decrease in TGF-β1–induced AT_{1}R expression by HGF (†P<0.01). Furthermore, to determine by which mechanism HGF controls PTEN synthesis, luciferase reporter constructs encoding the PTEN promoter were used. TGF-β1 significantly reduced PTEN reporter gene activity, whereas this effect was blocked by HGF treatment (Figure S3), suggesting that HGF regulated TGF-β1–induced PTEN transcriptional downregulation. These data indicated that HGF inhibited TGF-β1–induced AT_{1}R expression via the Akt signaling pathway.

**Overexpression of HGF Inhibited Renal Damage Induced by Ang II Infusion in HGF-Tg Mice**

To further confirm the in vitro findings, we used an in vivo model of Ang II infusion into mice, which exhibited severe renal damage. Our present data confirmed renal injury, such as fibrosis, by Ang II infusion (Figure 3). Because HGF is a renal protective growth factor, we used transgenic mice with cardiac-specific overexpression of human HGF (HGF-Tg). These HGF-Tg mice showed a significant increase in the serum human HGF concentration of ∼3-fold as compared with control littermates. As expected, HGF-Tg mice exhibited a much lower degree of renal injury, as assessed by diameter of the glomerulus, and glomerular injury index (Figure 3A through 3C; *P<0.01, respectively). Similarly, HGF-Tg mice exhibited significantly less renal fibrosis as compared with control (Figure 3D and 3E; *P<0.01). Although there was no significant difference in systolic blood pressure (SBP) between HGF-Tg and wild-type mice at baseline (SBP: 100.1 ± 2.8 versus 104.8 ± 5.8 mm Hg, respectively), SBP was significantly lower in HGF-Tg mice than in control littermates after 3 weeks of Ang II infusion (Figure S4; SBP: 104.2 ± 3.1 versus 120.1 ± 1.0 mm Hg, respectively; †P<0.05). Thus, to examine whether the prevention of renal fibrosis in HGF-Tg mice could be attributed to the reduction in BP, we further examined the effects of hydrazine, an antihypertensive drug, on fibrosis. Although treatment with hydralazine significantly decreased SBP and DBP as compared with vehicle treatment (Figure S4; SBP: 120.1 ± 1.8 versus 107.3 ± 4.1 mm Hg; DBP: 79.2 ± 4.7 versus 69.7 ± 4.2 mm Hg; *P<0.05, respectively), there were no significant differences in the diameter of glomeruli and glomerular injury index between control and hydralazine-treated animals (Figure 3). These data indicate that reductions in blood pressure alone may not be sufficient to account for the renal protective effects of HGF. To further explore the role of HGF in renal protection, we administered a neutralizing antibody against HGF to HGF-Tg mice treated with Ang II. At baseline, there was no difference in SBP between those with or without neutralizing antibody against HGF in HGF-Tg mice (100.1 ± 2.8 versus 99.8 ± 1.8 mm Hg, respectively). As shown in Figure 3, the renal protective effect in HGF-Tg mice was significantly inhibited by neutralizing anti-HGF antibody (Figure 3A through 3C; †P<0.01). Similarly, renal fibrosis was significantly inhibited in HGF-Tg mice as compared with control, whereas administration of neutralizing anti-HGF antibody attenuated the renal protective action in HGF-Tg mice (Figure 3D and 3E; †P<0.01).
Interestingly, Ang II infusion into control mice significantly increased the expression of renal AT1R mRNA as determined by quantitative PCR analyses (Figure 4A; \( P \leq 0.05 \)). In contrast, renal AT1R mRNA induced by Ang II infusion was significantly lower in HGF-Tg mice (Figure 4A; \( P \leq 0.05 \)). Because our previous report demonstrated that TGF-\( \beta \) mRNA expression was significantly lower in HGF-Tg mice as compared with control,\(^5\) we hypothesized that HGF might affect PTEN expression and Akt phosphorylation in the kidney, because PTEN attenuates Akt phosphorylation. As shown in Figure 4B, Ang II infusion to control mice induced a marked decrease in the level of PTEN and an increase in the level of Akt phosphorylation. However, in HGF-Tg mice treated with Ang II, PTEN gradually increased and Akt phosphorylation decreased. The contribution of HGF was confirmed by the observation that treatment with neutralizing antibody against HGF significantly decreased the increase in PTEN expression (Figure 5A and 5B; \( P \leq 0.05 \)). Importantly, neutralizing anti-HGF antibody significantly increased AT1R expression induced by Ang II, whereas AT1R expression induced by HGF was significantly lower in HGF-Tg mice as compared with control (Figure 5C; \( P \leq 0.05 \)).

Discussion

Mesangial cells are the major cells involved in the development of CKD. Ang II regulates mesangial cell hypertrophy and increases the expression and synthesis of ECM. Indeed, it is well known that blockade of the activation of RAS by ACE inhibitors and ARBs preserved or improved renal function in numerous clinical studies. Another important player related to CKD is TGF-\( \beta \), which is mainly involved in the progression of glomerulosclerosis. Thus, researchers tried to elucidate the relation between Ang II and TGF-\( \beta \). In mesangial cells, Ang II increased TGF-\( \beta \) mRNA expression, whereas a neutralizing antibody against TGF-\( \beta \) significantly reduced ECM production induced by Ang II.\(^{12}\) Furthermore, previous reports showed that ARBs inhibited the expression of TGF-\( \beta \).\(^{13–15}\) The present study demonstrated that TGF-\( \beta \) increased the expression of AT1R, suggesting positive feedback of Ang II-TGF-\( \beta \) by which Ang II increased TGF-\( \beta \), the induced TGF-\( \beta \) expression stimulated AT1R expression, and upregulation of AT1R enhanced Ang II signaling (Figure S5). To clarify this complicated pathway, we focused on PTEN, because PTEN protein is a phosphatidylinositol phosphate phosphatase specific for the 3-position ring. Although PTEN can dephosphorylate PI3P, PI(3,4)P2, and PI(3,4,5)P3, it is
likely that PI(3,4,5)P3 level in the plasma membrane decreases, which, in turn, regulates Akt kinase. Furthermore, a change in expression and activation of PTEN is involved in the pathophysiology of various diseases, such as pulmonary fibrosis. Moreover, PTEN acts as a negative regulator of myofibroblast, a major source of TGF-β. The present study clearly demonstrated that the missing link in the Ang II-TGF-β pathway is PTEN/Akt.

Interestingly, one of the most well-known factors antagonizing TGF-β is HGF. Currently, HGF is reported to counteract the profibrotic action of TGF-β1 by intercepting Smad signaling through different mechanisms in various types of kidney cells. For example, in human mesangial cells, HGF signaling stabilizes the Smad transcriptional corepressor TGF-β-induced factor homeobox 1 (TGIF) against degradation. Accumulated TGIF binds to Smads and sequesters their ability to initiate the transcription of TGF-β1 target genes.

In addition, our recent study demonstrated that HGF induced apoptosis of myofibroblasts, associated with an increase in MMPs expression and a decrease in ECM, potentially because of inhibition of TGF-β1 signaling. In addition to these known mechanisms, the present study suggests a novel pathway through the PI3K-Akt/PTEN pathway. HGF plays the role of antiapoptotic effect, mediating the activation of Akt/PI3K pathway. However, our present data showed that pretreatment with HGF decreased TGF-β1-induced Akt phosphorylation. In addition, some groups, other than ours have reported that HGF has antiapoptotic effects on endothelial cells. On the other hand, HGF induced apoptotic cell death in some distinct types of cells, including transformed or neoplastic cells and myofibroblasts. It might be a reason that HGF biologically regulates cell survival in a cell type–dependent manner. Furthermore, our recent study reported that HGF promotes oxidative stress in cultured endothelial cells but inhibits Ang II–induced excess oxidative stress thorough an epidermal growth factor receptor degradation mechanism. These results suggest that it might be different from the expression levels of c-Met, because local HGF is thought to be commonly decreased on pathological conditions. However, further studies are necessary to reveal the role of the c-Met/HGF system.

Here, we demonstrated that HGF inhibited the increase in AT1R expression by TGF-β1 by inhibition of phosphorylation of Akt through upregulation of PTEN. Not only in vitro experiments but also in vivo studies using HGF-Tg mice demonstrated that HGF inhibited the increase in AT1R expression by Ang II through the PI3K-Akt/PTEN pathway. From the clinical view points, it is noteworthy that ACE inhibitors and ARBs significantly increased local HGF expression in the heart, kidney, and other organs in various models. Our present data demonstrated that reductions in blood pressure alone may not be sufficient to account for the renal protective effects of HGF. Because ARBs and ACE inhibitors, which are known to increase local HGF expression, have pleiotropic effects beyond blood pressure control, the increase in local HGF expression by ARBs or ACE inhibitors might contribute to those pleiotropic effects. In fact, we confirmed that telmisartan, one of the ARBs that have the characteristics of a partial agonist of peroxisome proliferator-activated receptors, induced HGF expression in rat mesangial cells (data not shown).

Overall, the present study revealed novel molecular mechanisms of the inhibition of the Ang II-TGF-β-positive feedback system by HGF, which was related to the PI3K-Akt/PTEN pathway. The increase in local HGF by blockade of RAS might contribute to the clinical benefits of ARBs and ACE inhibitors in CKD. A clinical trial of recombinant HGF to prevent renal injury is now underway. Further development of pharmacotherapy to stimulate HGF, together with RAS inhibition, might improve CKD.

**Perspectives**

CKD is an independent risk factor for cardiovascular events and outcomes. Conversely, cardiovascular diseases are asso-
attenuation of renal fibrosis. Furthermore, our group and others showed an antifibrotic effect of HGF on not only renal fibrosis but also cardiac fibrosis. Activation of the HGF neutralizing antibody against HGF.

Figure 5. Effect of PTEN on angiotensin (Ang) II type 1 receptor (AT1R) expression under Ang II infusion in vivo. A. Immunoblotting analysis of PTEN expression in vivo. Hyd represents hydralazine. B. Quantification of immunoblotting of A. Data represent mean±SEM. *P<0.05 vs control with Ang II, †P<0.05 vs heparocyte growth factor (HGF)-transgene (Tg) without neutralizing antibody against HGF. C. AT1R mRNA expression in vivo. AT1R mRNA expression was determined by quantitative PCR using β-actin as an internal control. Data represent mean±SEM. *P<0.05 vs control with Ang II, †P<0.05 vs HGF-Tg without neutralizing antibody against HGF.

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Disclosures
None.

References


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Hepatocyte Growth Factor Attenuates TGF-β- Angiotensin II Crosstalk through Inhibition of PTEN/Akt Pathway

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Supplementary Materials and Methods

Materials
Human renal mesangial cells (HMC) and mesangial cell growth medium (MsBM) were purchased from Cambrex (Walkersville, MD). Antibody to anti-α-tubulin and Ang II were purchased from Sigma (St Louis, MO), and anti-phospho Akt antibodies and PTEN antibodies were purchased from Cell Signaling (Boston, MA). Antibodies to Akt1/2 (N-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN). Recombinant human HGF protein was purchased from Peprotech EC (London, UK). Phosphatidylinositol 3-kinase inhibitor (LY-294002), mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 inhibitor (PD-98059), p38 mitogen-activated protein kinase inhibitor (SB-203580), and c-Jun N-terminal kinase inhibitor (SP-600125) were purchased from Calbiochem (San Diego, CA). PTEN specific inhibitor (bpV(HO pic) was purchased from Enzo Life Sciences (Farmingdale, NY).

Evaluation of glomerular injury and expansion
Renal sections embedded in paraffin (5 µm thick) were stained with periodic acid-Schiff and examined with a light microscope. Glomerular injury scores were graded as 0: 0 to 10%; 1+: 10 to 25%; 2+: 26 to 50%; 3+: 51 to 75%; 4+: 75 to 100%1. Glomerulosclerotic expansion was scored on a scale of 0 to 4 as described by Raij et al. 2 These were then analyzed visually under a light microscope by two investigators.
blinded to treatment.
To evaluate of renal intersitital fibrosis, kidney sections (4-5 sections, 5 μm thick, per kidney) were stained with Masson's trichrome stain and analyzed visually under a light microscope by two investigators blinded to treatment. Masson's Trichrome-stained images were subsequently quantified using NIH ImageJ software. Sirius red staining was employed. Sections of kidney 5 μm thick were placed on slides, deparaffined and incubated with a saturated solution of picric acid containing 0.1 % Sirius red. Sirius red-stained images were also subsequently quantified using NIH ImageJ software.

**PTEN promoter-luciferase assay**

The PTEN vector was kindly provided by Prof. Mak TW\(^3\). For measuring luciferase activity, human mesangial cells were grown in 12-well plates. 0.2 ug Luciferase plasmid was co-transfected with 0.01 ug pGL4 Renilla plasmid (Promega) as control for the transfection efficiency using lipofectamine 2000 (invitrogen) according to the manufacturer’s protocol. The activity of Luciferase and Renilla was assessed with the Dual-Luciferase Reporter Assay System (Promega).

**Supplementary references**

Figure S1
Time course of AT1R mRNA expression induced by TGF-β1.

AT1R mRNA expression was determined by quantitative PCR using b-actin as an internal control. Data represent mean ± SEM, *: P<0.05; non-treated vs. TGF-b1-treated.
Figure S2
Time course of PTEN expression induced by TGF-β1 and effect of HGF.

(A) Immunoblotting analysis of PTEN expression after treatment with TGF-β1 in vitro (1 ng/ml for 1 to 3 hr). (B) Immunoblotting analysis of PTEN expression after treatment with TGF-β1 (1 ng/ml for 1 to 3 hr) and HGF (40 ng/ml) in vitro.
Figure S3
PTEN promoter assay

PTEN reporter gene activity after treatment with TGF-β1 (1 ng/ml for 1 hr) and/or HGF (40 ng/ml) in vitro. *P<0.05; vs no treatment group,
Figure S4
the evaluation of Ang II-induced blood pressure after 3 weeks in vivo.

Blood pressure was measured by the tail cuff method. Hyd represent hydrazine-treated mice. *P<0.05; vs control with Ang II
The model for vicious cycle of Ang II and TGF-β1.

First, Ang II increased TGF-β1 expression, and then TGF-β1 induced AT1R expression. Up-regulation of AT1R enhanced Ang II signaling cascade, resulting in the acceleration of renal fibrosis. HGF antagonizes TGF-β1 induced AT1R expression through the inhibition of PTEN.