Telmisartan Exerts Renoprotective Actions via Peroxisome Proliferator-Activated Receptor-\(\gamma\)/Hepatocyte Growth Factor Pathway Independent of Angiotensin II Type 1 Receptor Blockade

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Abstract—Angiotensin (Ang) II type 1 receptor blockers have demonstrated beneficial effects beyond blood pressure control in the treatment of chronic kidney disease. There is clinical evidence that telmisartan is more effective than losartan in reducing proteinuria in hypertensive patients with diabetic nephropathy, because it is a partial agonist of peroxisome-proliferator activated receptor-\(\gamma\) (PPAR\(\gamma\)), as well as an Ang II type 1 receptor blocker (AMADEO Study [A comparison of telMisartan versus losArtan in hypertensive type 2 DiabEtic patients with Overt nephropathy]). In this study, we examined the role of PPAR\(\gamma\) activation in the renal protective actions of telmisartan using Ang II type 1 receptor–deficient mice. Renal injury was induced in Ang II type 1 receptor–deficient mice by producing unilateral ureteral obstruction, which exhibited severe renal interstitial fibrosis and inflammation. In these mice, telmisartan prevented hydronephrosis induced by unilateral ureteral obstruction more strongly than did losartan. Importantly, the prevention of renal atrophy and fibrosis by telmisartan was significantly attenuated by GW9662, a PPAR\(\gamma\) antagonist. Interestingly, the downstream effector of PPAR\(\gamma\) activation by telmisartan is hepatocyte growth factor (HGF), a well-known antifibrotic factor, because renal HGF expression was significantly increased by telmisartan, and a neutralizing antibody against HGF diminished the renal protective action of telmisartan. These beneficial changes by telmisartan were associated with a decrease in the expression of transforming growth factor-\(\beta\)1 and other proinflammatory and profibrotic cytokine genes through PPAR\(\gamma\)/HGF activation. Our findings provide evidence of organ protective actions of telmisartan through the PPAR\(\gamma\)/HGF pathway, independent of Ang II type 1 receptor blockade. Further development of the next generation of Ang II type 1 receptor blockers with added organ protective actions, such as PPAR\(\gamma\) activation, might provide new beneficial drugs to treat renal and cardiovascular diseases. (Hypertension. 2012;59:308-316.) ● Online Data Supplement

Key Words: hepatocyte growth factor ■ angiotensin receptors ■ angiotensin antagonists ■ PPAR-\(\alpha\) and -\(\gamma\) ■ growth factors and cytokines ■ chronic failure (kidney) ■ hypertension (kidney)

Excessive activation of the renin-angiotensin (Ang) system, specifically Ang II, is a key component of the pathogenesis of hypertension, atherosclerosis, coronary artery disease, myocardial infarction, congestive heart failure, and nephropathy.\(^1\) Ang II exerts its effects through 2 different receptors, Ang II type 1 and type 2. The binding of Ang II to the type 1 receptor (AT\(_R\)) produces vasoconstriction, increases aldosterone release and sympathetic activity, and mediates virtually all of the known adverse cardiovascular effects of Ang II. AT\(_R\) blockers (ARBs) are selective antagonists of AT\(_R\) and are widely used to treat hypertension and hypertension-related target organ damage.\(^2\)–\(^5\) Diabetic nephropathy, the leading cause of end-stage renal disease, is a combination of hemodynamic and metabolic abnormalities that contribute to kidney damage resulting in proteinuria and reduced glomerular filtration rate.\(^6\) Among the available ARBs, telmisartan, one of the second-generation ARBs known as metabosartans, is reported to be more effective than losartan in reducing proteinuria in hypertensive patients with diabetic nephropathy, despite a similar reduction in blood pressure (AMADEO [A comparison of telMisartan versus losArtan in hypertensive type 2 DiabEtic patients with Overt nephropathy]).\(^7\) However, the mechanism explaining why telmisartan has better renal protective effects than losartan is not fully understood, although telmisartan is reported to have greater lipophilicity and a longer half-life than losartan.
A more reasonable explanation is that telmisartan is a partial agonist of peroxisome proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\)).\(^8,9\) because PPAR\(\gamma\) is a nuclear receptor transcription factor that binds to a putative peroxisome proliferator response element, leading to activation of various genes related to many important physiological processes, including adipose differentiation and lipid and glucose metabolism.\(^10\) Recently, pioglitazone, a synthetic ligand of PPAR\(\gamma\) that is widely used in the treatment of type 2 diabetes mellitus, was demonstrated to reduce cardiovascular events and proteinuria and to delay the progression of diabetic nephropathy, independent of glycemic control.\(^11,12\) There is substantial evidence that activation of PPAR\(\gamma\) leads to suppression of proinflammatory and proatherogenic molecules. Therefore, combined AT\(_1\)R antagonism and PPAR\(\gamma\) activation, such as by telmisartan, would be expected to effectively treat the metabolic and inflammatory derangements associated with the metabolic syndrome, which contribute to the development of atherosclerosis, myocardial fibrosis, and glomerulosclerosis. Among numerous downstream effectors of PPAR\(\gamma\) activation, we focused on hepatocyte growth factor (HGF), because peroxisome proliferator response element exists in the promoter region of the HGF gene, which increases local HGF gene expression.\(^13\) Moreover, HGF is well known to prevent the initiation and progression of chronic renal fibrosis and to inhibit the expression and signal of transforming growth factor-\(\beta\) (TGF-\(\beta\)) in various animal models.\(^14\)–\(^18\) Recently, our previous study showed that HGF attenuated renal fibrosis through apoptosis of myofibroblasts via TGF-\(\beta\)1 suppression.\(^19\) To elucidate the role of PPAR\(\gamma\) activation, HGF, and TGF-\(\beta\) in the organ protective actions of telmisartan, we used a hydronephrosis model produced by unilateral ureteral obstruction (UUO) in AT\(_{1\alpha}\)-deficient mice. Here, we showed evidence of organ-protective actions of telmisartan through the PPAR\(\gamma\)/HGF pathway, independent of AT\(_1\)R blockade.

Materials and Methods

Experimental UUO Model in AT\(_{1\alpha}\)-R-Deficient Mice

UUO was performed on 8-week-old male AT\(_{1\alpha}\)-R-deficient mice (Jackson Laboratory, Bar Harbor, ME) after 1 week of pretreatment with drugs, according to established procedures. Under general anesthesia, losartan (3 mg/kg per day), (d) UUO-operated mice treated with telmisartan (3 mg/kg per day), (e) UUO-operated mice treated with telmisartan and neutralizing hepatocyte growth factor neutralizing antibody (HGF-Ab; 200 \(\mu\)g/wk), and (f) UUO-operated mice treated with losartan, (d) UUO-operated mice treated with telmisartan, (e) UUO-operated mice treated with telmisartan and HGF-Ab, and (f) UUO-operated mice treated with telmisartan and HGF-Ab. Bar: 1 mm. B. Typical micrographs of kidney with acid-Schiff staining after UUO; (a) sham-operated mice, (b) UUO-operated mice (control), (c) UUO-operated mice treated with HGF-Ab, (d) UUO-operated mice treated with telmisartan and GW9662 (a peroxisome-proliferator activated receptor [PPAR]-\(\gamma\) antagonist, 0.3 mg/kg per day). Bar: 100 \(\mu\)m. C. Quantitative data for percentage of fibrotic area. Sham indicates sham-operated; –, UUO-operated mice (control); Los, losartan; Tel, telmisartan; GW, GW9662. *\(P<0.05\) vs control, †\(P<0.05\) vs Tel. Data are shown as mean±SEM (n=5).

Figure 1. Renal remodeling and interstitial fibrosis at 14 days after unilateral ureteral obstruction (UUO) operation. A. Typical micrographs of periodic cross-sections of kidney with acid-Schiff staining after UUO; (a) sham-operated mice, (b) UUO-operated mice (control), (c) UUO-operated mice treated with telmisartan, (d) UUO-operated mice treated with losartan, (c) UUO-operated mice treated with telmisartan and GW9662 (a peroxisome-proliferator activated receptor [PPAR]-\(\gamma\) antagonist, 0.3 mg/kg per day). Bar: 1 mm. B. Typical micrographs of kidney with acid-Schiff staining after UUO; (a) sham-operated mice, (b) UUO-operated mice (control), (c) UUO-operated mice treated with telmisartan and GW9662 (a peroxisome-proliferator activated receptor [PPAR]-\(\gamma\) antagonist, 0.3 mg/kg per day). Bar: 1 mm. B. Typical micrographs of kidney with acid-Schiff staining after UUO; (a) sham-operated mice, (b) UUO-operated mice (control), (c) UUO-operated mice treated with telmisartan and GW9662 (a peroxisome-proliferator activated receptor [PPAR]-\(\gamma\) antagonist, 0.3 mg/kg per day). Bar: 1 mm. B. Typical micrographs of kidney with acid-Schiff staining after UUO; (a) sham-operated mice, (b) UUO-operated mice (control), (c) UUO-operated mice treated with telmisartan and GW9662 (a peroxisome-proliferator activated receptor [PPAR]-\(\gamma\) antagonist, 0.3 mg/kg per day). Bar: 1 mm.
anesthesia, complete ureteral obstruction was performed by double ligation of the left ureter with 5-0 silk after midline abdominal incision. Sham-operated mice had their ureters exposed and manipulated but not ligated. Mice were divided into 11 groups: (1) sham, (2) UUO-operated mice with no drug treatment (control), (3) UUO-operated mice treated with losartan (3.0 mg/kg per day), (4) UUO-operated mice treated with telmisartan (3.0 mg/kg per day), (5) UUO-operated mice treated with telmisartan (3.0 mg/kg per day) and neutralizing HGFA antibody (HGFA-Ab; 200 mg/wk), (6) UUO-operated mice treated with telmisartan (3 mg/kg per day) and neutralizing HGFA-Ab (200 μg/wk), (7) UUO-operated mice treated with losartan (3.0 mg/kg per day) and neutralizing HGFA-Ab (200 μg/wk), (8) UUO-operated mice treated with losartan (3.0 mg/kg per day) and GW9662 (0.3 mg/kg per day), (9) UUO-operated mice treated with telmisartan (10 mg/kg per day), (10) UUO-operated mice treated with losartan (6.0 mg/kg per day), and (11) UUO-operated mice treated with losartan (10 mg/kg per day; 5–7 mice for each group).

Drugs were dissolved in water and administered ad libitum. Mice were housed in the animal facilities of Osaka University with free access to food and water. Mice were euthanized at 2 weeks after operation.

### Cell Isolation and Culture

In in vitro experiments, dermal fibroblasts obtained from AT1aR-deficient mice were used. Dermal fibroblasts were obtained using a dermapunch (Maruho, Osaka, Japan) as instructed by the manufacturer, followed by culture in DMEM (Nacalai, Kyoto, Japan) plus 10% FBS (Sanko Junyaku, Tokyo, Japan) and antibiotics and incubation at 37°C in a humidified 5% CO2 and 95% air atmosphere. Studies were performed by growing the cells to 80% confluence and rendering them quiescent by replacing the complete medium with serum-free medium for 24 hours and then incubating them in incremental concentrations of TGF-β1 (1 ng/mL). Cells were pretreated with drugs (telmisartan: 10 μmol/L, losartan: 10 μmol/L, HGFA-Ab: 10 μmol/L, and GW9662: 10 μmol/L) for 12 hours before TGF-β1 stimulation. Western blotting was used to examine the cells for expression of α-smooth muscle actin (SMA) protein induced by TGF-β1. Lysates of the cells were obtained after 48 hours of TGF-β1 stimulation. Western blotting was performed using primary antibodies, mouse monoclonal anti–α-SMA, and anti–β-actin (Sigma-Aldrich, St Louis, MO).

There is more information about materials and methods in the Materials and Methods section of the online Data Supplement, available at http://hyper.ahajournals.org.

### Statistical Analysis

Data are expressed as mean±SEM. Comparisons were made using ANOVA followed by Turkey simultaneous multiple comparisons. Values with P<0.05 were considered significant.

### Results

#### Prevention of Hydronephrosis by Telmisartan Is Stronger Than That by Losartan, in AT1a,R-Deficient Mice

In this study, to elucidate the role of PPARγ activation by telmisartan, we used a renal injury model using AT1a,R-deficient mice, because traditional ARBs theoretically fail to demonstrate organ protective actions in the absence of AT1R. To induce renal injury, we generated a UUO model, because this model exhibits severe renal interstitial fibrosis and inflammation in the kidney on the obstructed side at 14 days after UUO operation. Indeed, our present study clearly demonstrated the typical appearance of renal atrophy in control mice after UUO operation (Figure 1A). As expected, losartan-treated kidneys showed more marked hydronephrosis than that in telmisartan-treated kidneys but seemed to show less marked hydronephrosis than that in untreated UUOs. Losartan also seemed to exert renoprotective effects to prevent hydronephrosis, but such effects were weaker than those of telmisartan (Figure 1A and Figure S1, available in the online Data Supplement). Prevention of hydronephrosis by telmisartan was also confirmed by measurement of the fibrotic area in obstructed kidneys (Figure 1B and 1C). In the obstructed kidneys of AT1a,R-deficient mice treated with losartan, Masson trichrome staining showed areas of severe renal fibrosis similar to control (Figure 1B and 1C). In contrast, quantification of renal fibrosis demonstrated a significant reduction in fibrotic area in the telmisartan-treated group, as compared with the losartan-treated group and control group (Figure 1C; P<0.05). There were no significant differences in blood pressure, heart rate (Table S2), serum creatinine level, and serum urea nitrogen among the groups (Table S3). These data clearly demonstrated the potential contribution of PPARγ activation, because telmisartan stimulated PPARγ activity separately from AT1R blockade. This hypothesis is further supported by the observation that the prevention of renal atrophy and fibrosis by telmisartan in the UUO model of AT1a,R-deficient mice was significantly attenuated by treatment with GW9662 (a PPARγ antagonist; Figure 1C and Figure S1; P<0.05).

To further explore the role of PPARγ activation, we focused on the downstream effectors of PPARγ activation.
Thus, we measured renal HGF concentration, because the promoter region of the HGF gene contains peroxisome proliferator response element. Importantly, protein expression of HGF was significantly increased in the obstructed kidneys of AT\(_1\)R-deficient mice treated with telmisartan, whereas losartan had no significant effect (Figure 2A; \(P<0.01\)). Similarly, serum HGF concentration was also significantly increased in mice treated with telmisartan (Figure 2B; \(P<0.01\)). The increase in HGF expression by telmisartan was attributed to PPAR\(\gamma\) activation, because treatment with GW9662 significantly decreased renal and serum HGF concentrations (Figure 2; \(P<0.01\)). Because HGF is well known to inhibit fibrosis, we administered a neutralizing antibody against HGF (HGF-Ab). As shown in Figure 1A through 1C, HGF-Ab significantly diminished the renal protective action of telmisartan (\(P<0.05\)). These data suggest that telmisartan exerts additional renal protective actions through PPAR\(\gamma\)/HGF activation, separate from AT\(_1\)R blockade, more strongly than does losartan.

**Molecular Mechanisms of Organ-Protective Actions of Telmisartan-PPAR\(\gamma\)/HGF Activation in AT\(_1\)R-Deficient Mice**

Next, we histologically evaluated renal injury by acid-Schiff staining. Glomerular changes were observed in the UUO model, characterized by lipid deposits in the mesangium and capillaries and glomerular hyperplasia with deposition of extracellular matrix in the mesangium. Tubulointerstitial changes including macrophage infiltration and a modest increase in the interstitial space as a result of extracellular matrix (ECM) deposition: (a) sham, sham-operated mice; (b) UUO-operated mice treated with losartan (3 mg/kg per day); (c) UUO-operated mice treated with telmisartan and neutralizing hepatocyte growth factor neutralizing antibody (HGF-Ab; 200 \(\mu\)g/wk), and (d) UUO-operated mice treated with telmisartan and GW9662 (a peroxisome-proliferator activated receptor [PPAR]-\(\gamma\) agonist, 0.3 mg/kg per day). Bar: 50 \(\mu\)m. B, Summary of glomerular injury index. Sham indicates sham-operated; –, UUO-operated mice (control); Los, losartan; Tel, telmisartan; GW, GW9662. \(*P<0.05, **P<0.01\) vs control, \(\dagger P<0.05\) vs Tel. Data are shown as mean±SEM (n=5). C, Typical micrographs of renal tubulointerstitial sections with acid-Schiff staining. Data show tubulointerstitial injury including macrophage infiltration and increased interstitial space as a result of extracellular matrix deposition: (a) sham, sham-operated mice; (b) UUO-operated mice (control); (c) UUO-operated mice treated with losartan (3 mg/kg per day); (d) UUO-operated mice treated with telmisartan (3 mg/kg per day); (e) UUO-operated mice treated with telmisartan and neutralizing HGF-Ab (200 \(\mu\)g/wk); (f) UUO-operated mice treated with telmisartan and GW9662 (a PPAR-\(\gamma\) agonist, 0.3 mg/kg per day). Bar: 100 \(\mu\)m. D, Summary of tubulointerstitial injury score. \(*P<0.01\) vs control, \(\dagger P<0.05\) vs Tel. Data are shown as mean±SEM (n=5).
ure 3A through 3C). In contrast, glomerular injury index and tubulointerstitial injury score were significantly reduced in the telmisartan group as compared with the losartan group and control group (Figure 3; \( P < 0.05 \)). Treatment with HGF-Ab, as well as GW9662, significantly diminished the decrease in glomerular injury index and tubulointerstitial injury score by telmisartan (\( P < 0.05 \)).

An early event in the development of tubulointerstitial fibrosis is the recruitment of inflammatory cells like macrophages. In the obstructed kidney at 14 days after UUO surgery, macrophage infiltration was observed in the renal tubulointerstitial area, as assessed by the F4/80-positive area. As shown in Figure 4A and Figure S2A, prevention of macrophage infiltration by telmisartan is stronger than that by losartan (\( P < 0.01 \)), although losartan also seemed to prevent macrophage infiltration, but such effects were weaker than those of telmisartan. Consistently, treatment with HGF-Ab, as well as GW9662, significantly diminished the decrease in the area of macrophage infiltration by telmisartan (\( P < 0.01 \)). More exciting findings of the present study were the expression of \( \alpha \)-SMA, which is the molecular hallmark of myofibroblasts, with localization and quantification of myofibroblasts, denoting the intensity of the fibrogenic response. Renal interstitial myofibroblast cells are \( \alpha \)-SMA–positive activated matrix-producing cells responsible for relentless accumulation and deposition of extracellular matrix in the interstitial compartment of diseased kidneys. As shown in Figure 4B and Figure S2B, telmisartan treatment resulted in a significant decrease in interstitial \( \alpha \)-SMA expression as compared with losartan treatment or control (\( P < 0.01 \)), whereas treatment with HGF-Ab, as well as GW9662, significantly diminished the decrease in interstitial \( \alpha \)-SMA expression induced by telmisartan (\( P < 0.01 \)). These beneficial changes by telmisartan were associated with a decrease in renal expression of TGF-\( \beta \)-1, a fibrogenic cytokine in the obstructed kidney. An increase in TGF-\( \beta \)-1 protein was detected mainly in the tubular epithelial cells of the obstructed kidney in control mice. However, telmisartan treatment significantly reduced interstitial TGF-\( \beta \)-1 expression as compared with the losartan-treated group and control group (Figure 4C and Figure S2C; \( P < 0.01 \)). The decrease in TGF-\( \beta \)-1 expression by telmisartan was attributed to PPAR\( \gamma \)/HGF activation, because HGF-Ab, as well as GW9662, attenuated the decrease in TGF-\( \beta \)-1 expression induced by telmisartan (\( P < 0.01 \)).

**Renoprotective Effects of Losartan Were Also Inhibited by HGF-Ab and GW9662**

In this model of kidney injury, losartan might exert beneficial effects on some parameters, but such effects would be weaker than those of telmisartan. The weak PPAR\( \gamma \) agonistic effects of losartan may affect the antifibrotic and anti-inflammatory actions of losartan. When HGF-Ab and GW9662 were administered together with losartan, the degrees of glomerular injury and of macrophage infiltration observed were similar to those in mice receiving no drugs (Figure S3). Therefore, it is supposed that the weak PPAR\( \gamma \) agonistic effects of losartan may induce the beneficial changes seen in these models.

**Renoprotective Effects of Each Drug Are Not Dependent on Dose**

If both losartan and telmisartan have similar effects on renal protection via the same pathway, a higher dose of losartan may test just as effective as telmisartan. Therefore, experiments were performed to test that telmisartan is more effective than losartan even when higher doses of losartan are used. Administration of 10 mg/kg per day of losartan showed no significant improvement in renal fibrosis, macrophage infiltration, glomerular injury index, and tubulointerstitial
Telmisartan Inhibited TGF-β1–Mediated Phenotype Change in AT1aR-Deficient Fibroblasts More Significantly Than Losartan

Finally, to identify the relationship between HGF and TGF-β1 in phenotype modulation, we performed in vitro experiments using dermal fibroblasts from AT1aR-deficient mice, because TGF-β1 initiates a phenotypic response in tubular epithelial cells to undergo epithelial–mesenchymal transition (EMT), becoming fibroblasts that migrate to the interstitium. Consistent with previous reports,21 TGF-β1 significantly induced a myofibroblast phenotype, as indicated by increased α-SMA protein expression (Figure 5A; P<0.01). Telmisartan, but not losartan, significantly inhibited the increase in expression of α-SMA induced by TGF-β1 (Figure 5A; P<0.05). We examined the role of HGF in myofibroblast phenotype change. As expected, HGF-Ab significantly reduced the inhibition of α-SMA expression by telmisartan (Figure 5B; P<0.05). As shown in Figure 6A, telmisartan significantly increased HGF expression in fibroblasts from AT1aR-deficient mice (P<0.01), whereas losartan did not increase HGF. The increased expression of HGF by telmisartan was significantly diminished by GW9662, consistent with in vivo data (Figure 6A; P<0.05). In addition, although TGF-β1 significantly increased profibrotic and profibrotic cytokine genes, such as monocyte chemoattractant protein 1, interleukin 6, collagen I, collagen IV, and plasminogen activator inhibitor 1 (P<0.01), telmisartan significantly inhibited the enhanced expression of these cytokines induced by TGF-β1 (Figure 6B through 6F; P<0.05). However, as shown in in vivo studies, losartan also inhibited the enhanced expression of some of these parameters (collagen I, collagen IV, and plasminogen activator inhibitor 1; Figure 6D through 6F; P<0.05), but such effects are weaker than those of telmisartan. The contribution of PPARγ/HGF activation was also confirmed by the observation that both HGF-Ab and GW9662 significantly attenuated the decrease in expression of these cytokines by telmisartan (Figure 6B through 6F; P<0.05).

Discussion

Using a UUO renal injury model in AT1aR-deficient mice, the present study provided direct evidence of renal protective actions of telmisartan through the PPARγ/HGF pathway, independent of AT1,R blockade. In this study, telmisartan, but not losartan, prevented tubulointerstitial fibrosis and activation of myofibroblasts (expressing α-SMA) and macrophages, which play a pivotal role in the progression of renal obstructive injury after UUO.22 Especially because tubulointerstitial fibrosis is an integral part of the structural changes in the kidney in chronic progressive renal failure,23 the present study is important for clinical practice.

In general, ARBs have beneficial effects on proteinuria and renal dysfunction that appear to be mediated by factors additional to their effects on blood pressure. The most intriguing question is whether there are some differences in organ protection among various ARBs, because telmisartan and irbesartan are multifunctional ARBs or “metabosartans” that activate PPARγ.24

Figure 5. Western blotting analysis of α-smooth muscle actin (α-SMA). Effects of transforming growth factor (TGF)-β1 (1 ng/mL), telmisartan (10 μmol/L), losartan (10 μmol/L), and hepatocyte growth factor neutralizing antibody (HGF-Ab; 10 nmol/L) on α-SMA protein in dermal fibroblasts from angiotensin II type 1 receptor–deficient mice. A, Top, Typical example of Western blotting for comparing telmisartan and losartan. Bottom, Quantitative analysis of Western blotting. Bar graphs show α-SMA/β-actin ratio. *P<0.01 vs control, †P<0.05 vs telmisartan. Data are shown as mean±SEM (n=4). B, Top, Typical example of Western blotting for evaluating the effects of HGF-Ab. Bottom, Quantitative analysis of Western blotting. Bar graphs show α-SMA/β-actin ratio. *P<0.01 vs control, †P<0.05 vs telmisartan. Data are shown as mean±SEM (n=4).
Based on the present study, the prevention of renal fibrosis and injury, at least in hydronephrosis, by telmisartan is mediated by PPARγ activation, leading to a local increase in HGF and a decrease in TGF-β, associated with the inhibition of the transdifferentiation of tubular epithelial cells into myofibroblasts, an event underlying progressive chronic kidney disease. HGF has been shown to exhibit a marked ability to block this phenotypic transition both in vitro and in vivo.14 HGF was also shown to ameliorate renal fibrosis and preserve kidney function in some chronic kidney disease models.15,16 In contrast, among many fibrogenic factors that regulate fibrotic processes, TGF-β is a strong inducer of transdifferentiation in renal tubular cells. Under the stimulus of TGF-β1 produced by macrophages or other cells, fibroblasts synthesize stress fibers and differentiate further into myofibroblasts.20 Myofibroblasts are contractile and augment the deposition of the extracellular matrix, leading to progressive interstitial fibrosis and the structural changes within the kidney in chronic progressive renal failure.25 A previous study demonstrated that HGF prevented the initiation and progression of chronic renal fibrosis and inhibited TGF-β1 expression in a wide variety of animal models.14 On the other hand, PPARγ agonists also significantly reduced TGF-β and attenuated renal interstitial fibrosis and inflammation in models of UUO.26 Thiazolidines have been proven to mediate anti-inflammatory effects and antifibrotic effects in animal models of diabetic nephropathy,27 consistent with several clinical studies in which thiazolidines reduced urine albumin excretion and prevented the development of renal injury.1,12,28 The present in vivo study demonstrated that the

Figure 6. Real-time PCR analysis. Effects of transforming growth factor (TGF)-β1 (1 ng/mL), telmisartan (10 μmol/L), losartan (10 μmol/L), GW9662 (10 μmol/L), and HGF neutralizing antibody (HGF-Ab; 10 nmol/L) on A, hepatocyte growth factor (HGF)/GAPDH mRNA relative expression; B, monocyte chemoattractant protein (MCP)-1/GAPDH mRNA relative expression; C, interleukin (IL)-6/GAPDH mRNA relative expression; D, collagen I/GAPDH mRNA relative expression; E, collagen IV/GAPDH mRNA relative expression; and F, plasminogen activator inhibitor (PAI)-1/GAPDH mRNA relative expression in dermal fibroblasts from angiotensin II type 1a receptor–deficient mice. *P<0.01 vs no treatment, †P<0.05 vs TGF-β1, ‡P<0.01 vs TGF-β1. Data are shown as mean±SEM (n=4).
increase in HGF by PPARγ activation mediated by telmisartan prevented renal injury and fibrosis, associated with a decrease in TGF-β1. These effects of PPARγ activation by telmisartan were also confirmed by in vitro experiments using fibroblasts from AT1aR-deficient mice. Similarly, in fibroblasts derived from AT1aR-deficient mice, mRNA of inflammatory cytokines, such as monocyte chemoattractant protein 1 and interleukin 6, and profibrotic cytokines, such as plasminogen activator inhibitor 1, collagen I, and collagen IV, were upregulated by TGF-β1, whereas telmisartan reduced the increase in expression of these genes mediated by TGF-β1. Importantly, these effects of telmisartan were inhibited by a neutralizing HGF antibody and GW9662, a PPARγ antagonist.

There are some limitations of this study. The mice used in this study were AT1aR-deficient mice. In the losartan group, the area positively immunostained for F4/80 and TGF-β1 was attenuated compared with that in the control group. Also, it has already been proven that losartan has weak PPARγ agonistic activity as well. We chose AT1aR-deficient mice, so the AT1bR-blocking action of losartan may have anti-inflammatory effects. Crowley et al. showed previously that AT1b receptor activation increased podocyte injury and expression of inflammatory mediators using AT1bR-deficient C57Bl/6j mice. Also, administration of losartan reduced markers of kidney disease, such as proteinuria, glomerular pathology, and inflammatory cytokine expression. Also in this model, losartan might have beneficial effects on kidney injury (glomerular injury and macrophage infiltration, etc.), but such effects are weaker than those of telmisartan. These beneficial effects induced by losartan were attenuated by cotreatment with HGF-Ab or GW9662, and it is supposed that the weak PPARγ agonistic effects of losartan may induce the beneficial changes seen in these models. AT1aR-blocking action of losartan may not play the crucial role, even if it exists. In the kidney, it has been reported that AT1b receptor expression is largely limited to the glomerulus. Moreover, Zhou et al. reported that, in mouse kidney, the AT1a receptor is the predominant subtype of AT1R. These previous reports support our results. Losartan might also have anti-inflammatory effects in cultured fibroblasts derived from AT1aR-deficient mice, but such effects would be weaker than those of telmisartan as shown in in vivo studies. The same mechanism may operate in in vitro studies as seen in in vivo studies.

Moreover, even when the administration dosage of losartan was increased, the renal protective effects were not changed significantly. Therefore, the renal protective effects of these drugs appear to be independent of their administration dosage.

Another potential limitation of this study is the use of the tail-cuff technique to measure blood pressure. The tail-cuff technique does not give 24-hour blood pressure, blood pressure variability, and so forth. Thus, it is possible that some of the greater renoprotective effects of telmisartan versus losartan might have been because of greater effects of telmisartan on 24-hour blood pressure or other components of blood pressure not measured by the tail-cuff technique.

In conclusion, the present study provides direct evidence of organ-protective actions of telmisartan through the PPARγ/HGF pathway, independent of AT1R blockade. Losartan also provided the similar effects, but those effects are weaker than those of telmisartan.

Because inhibition of the renin-Ang system does not control all of the pathophysiological mechanisms of hypertension or cardiovascular risk, the development of the next generation of ARBs with added organ protective actions that can target additional mechanisms of hypertension, cardiovascular disease, and diabetes mellitus is clearly important. Indeed, next-generation ARBs that are intended to not only antagonize AT1R but also to block endothelin receptors, function as NO donors, inhibit neprilysin activity, and increase natriuretic peptide level or stimulate PPARγ are now becoming available.

Perspectives

In AT1aR-deficient mice with unilateral ureteral obstruction, we have found that the renoprotective actions of telmisartan may not simply depend on AT1 receptor blockade and may be partially mediated by its ability to activate PPARγ and increase the expression of HGF. HGF appears to be an important downstream effector molecule of the organ-protective effects of PPARγ agonists. To the extent that the renal protective effects of telmisartan extend beyond the UUO model, it is possible that ARBs that activate PPARγ and stimulate HGF may someday prove useful in limiting the progression of a variety of forms of chronic kidney disease. Further drug design to add PPARγ activation to ARBs might provide new beneficial drugs to treat renal and cardiovascular disease in diabetes mellitus, obesity, and the metabolic syndrome.

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Disclosures

None.

References

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Telmisartan Exerts Renoprotective Actions via PPARγ/HGF Pathway Independent of Angiotensin II type 1 Receptor Blockade

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Supplementary Materials and Methods

Materials

Telmisartan was donated by Boehringer Ingelheim (Ingelheim, Germany). Losartan was purchased from LKT Laboratories Inc. (St Paul, USA). HGF neutralizing antibody was purchased from Kringle Pharma (Osaka, Japan). All procedures were approved by the Animal Use and Care Committee of Osaka University. Systolic BP and heart rate (HR) were measured by the tail-cuff method (UR-5000: Softron, Tokyo, Japan).

Immunohistochemical staining

Immunohistochemical staining was performed on tissues fixed with 4% formalin and embedded in paraffin, as described previously. Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN). Immunostaining of α-SMA, F4/80 and TGF-β1 was performed using mouse anti-mouse α-SMA antibody (M0851: DAKO, Glostrup, Denmark), mouse anti-mouse F4/80 antibody (ab6640: Abcam, Cambridge,
UK), and mouse anti-mouse TGF-β1 antibody (sc-146: Santa Cruz, Santa Cruz, USA), respectively. Immunostained images were quantified using NIH Image J software (http://rsb.info.nih.gov/ij/), and then analyzed visually under a light microscope by two investigators blinded to treatment.

**Evaluation of glomerular and tubulointerstitial injury and renal fibrosis**

Renal sections embedded in paraffin (5 μm thick) were stained with periodic acid-Schiff and examined by light microscopy. Tubulointerstitial injury scores were graded as 0, 0 to 10%; 1+, 1 to 25%; 2+, 26 to 50%; 3+, 51 to 75%; 4+, 75 to 100%, as described previously. 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%, as described by Raij et al. They were then analyzed visually under a light microscope by two investigators blinded to treatment.

To evaluate renal 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 Image J software (http://rsb.info.nih.gov/ij/).

**Measurement of HGF**

HGF concentration was measured by an enzyme linked immunosorbent assay, using an IMMUNIS mouse HGF EIA kit and MMUNIS HGF extraction buffer (Institute of Immunology Co., Ltd., Tokyo, Japan). Mouse kidney samples were disintegrated with IMMUNIS HGF extraction buffer using a Multi-beads shaker (Yasui Kikai, Osaka, Japan) at 2000g for 15 seconds. Homogenates were centrifuged at 14,000g for 30 min. The supernatant was used for HGF assay, according to the manufacturer’s instructions.
Quantitative real-time polymerase chain reaction

Total RNA was extracted from cultured cells, and quantitative real-time PCR was performed with an ABI Prism 7000 Sequence Detection System using PCR Master Mix Reagent (Applied Biosystems, Foster, USA). Total RNA was extracted after 24 hours of TGF-β1 stimulation, as described previously. In each experiment, mouse GAPDH RNA was amplified as a reference standard. Primers used are listed in Table S1. Levels of mRNA were normalized to that of GAPDH mRNA.

Supplementary references


Figure S1.

Quantification data of renal atrophy assessed by parenchymal area / whole cross-sectional area (%)

*P<0.05 vs. mice treated with telmisartan (3 mg/kg/day).
†P<0.05 vs. control mice. Data are shown as mean ±SEM (n=5).
Sham; sham-operated, (-); UUO-operated mice (Control), Los; losartan, (-); UUO-operated mice (Control),
Tel; telmisartan, HGF-Ab; HGF neutralizing antibody, GW; GW9662.
Figure S2. Immunohistochemical staining for F4/80, α-SMA and TGF-β1.

A, Immunohistochemical staining for F4/80
B, for α-SMA
C, and for TGF-β1

Brown-stained area shows F4/80 protein-positive area, α-SMA protein-positive area and TGF-β1 protein-positive area, respectively.(a) Sham; sham-operated mice (b) (-); UUO-operated mice (Control), (c) UUO-operated mice treated with losartan (3 mg/kg/day), (d) UUO-operated mice treated with telmisartan (3 mg/kg/day), (e) UUO-operated mice treated with telmisartan and neutralizing HGF-Ab (200 mg/week), (f) UUO-operated mice treated with telmisartan and GW9662 (a PPARγ antagonist, 0.3 mg/kg/day). Bar=100 mm.
Figure S3.

A (a) (b) (c) (d)

B (e) (f) (g) (h)

Control (-) HGF-Ab GW9662

Losartan

C

Glomerular injury index

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>(-)</th>
<th>HGF-Ab</th>
<th>GW9662</th>
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<td>Glomerular injury index</td>
<td>†</td>
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D

% Positive area for macrophages (F4/80)

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<th></th>
<th>Control</th>
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<th>HGF-Ab</th>
<th>GW9662</th>
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<tr>
<td>% Positive area for macrophages (F4/80)</td>
<td>†</td>
<td></td>
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</tbody>
</table>
**Figure S3.**

**Renoprotective effects of losartan were also reduced by HGF-Ab and GW9662.**

A, Typical micrographs of renal glomerular sections with acid-Schiff staining to evaluate mesangial proliferation; (a) Control: UUO-operated mice, (b) (-); UUO-operated mice treated with losartan (3 mg/kg/day), (c) UUO-operated mice treated with losartan (3 mg/kg/day) and neutralizing HGF-Ab (200 mg/week), (d) UUO-operated mice treated with losartan (3 mg/kg/day) and GW9662 (PPAR\_\gamma antagonist, 0.3 mg/kg/day). Bar=50 \( \mu m \).

B, Typical micrographs of immunohistochemical staining for macrophages (F4/80); (e) Control: UUO-operated mice, (f) UUO-operated mice treated with losartan (3 mg/kg/day), (g) UUO-operated mice treated with losartan (3 mg/kg/day) and neutralizing HGF-Ab (200 mg/week), (h) UUO-operated mice treated with losartan (3 mg/kg/day) and GW9662 (PPAR\_\gamma antagonist, 0.3 mg/kg/day). Bar=100 \( \mu m \).

C, Summary of glomerular injury index

D, Quantitative data of macrophage infiltration area (%).

Control: UUO-operated mice, (-); UUO-operated mice treated with losartan, HGF-Ab; UUO-operated mice treated with losartan and neutralizing HGF-Ab, GW9662; UUO-operated mice treated with losartan and GW9662 †P<0.05 vs. treatment with only losartan. Data are shown as mean \( \pm \) SEM (n=5).

**Figure S4.**

**Renal fibrosis and macrophage infiltration at each dosage of telmisartan and losartan**

A, Typical micrographs of kidney with Masson’s trichrome staining. Blue color shows fibrotic areas (collagen and fibronectin deposition); (a) UUO-operated mice treated with telmisartan (3 mg/kg/day), (b) UUO-operated mice treated with telmisartan (10 mg/kg/day), (c) UUO-operated mice treated with losartan (3 mg/kg/day), (d) UUO-operated mice treated with losartan (6 mg/kg/day), (e) UUO-operated mice treated with losartan (10 mg/kg/day). Bar=100 mm.

B, Quantitative data for % fibrotic area †P<0.05 vs. mice treated with telmisartan (3 mg/kg/day). Data are shown as mean \( \pm \) SEM (n=5).

C, Typical micrographs of immunohistochemical staining for macrophages (F4/80); (a) UUO-operated mice treated with telmisartan (3 mg/kg/day), (b) UUO-operated mice treated with telmisartan (10 mg/kg/day), (c) UUO-operated mice treated with losartan (3 mg/kg/day), (d) UUO-operated mice treated with losartan (6 mg/kg/day), (e) UUO-operated mice treated with losartan (10 mg/kg/day). Bar=100 \( \mu m \).

D, Quantitative data of macrophage infiltration area (F4/80), Los; losartan, Tel; telmisartan. †P<0.01 vs. mice treated with telmisartan (3 mg/kg/day).

Data are shown as mean \( \pm \) SEM (n=5).
Figure S4.

A

(a) Telmisartan 3 mg/kg/day (mg/kg/day)
(b) Telmisartan 10 mg/kg/day

3 10

Telmisartan

(c) Losartan 3 mg/kg/day
(d) Losartan 6 mg/kg/day
(e) Losartan 10 mg/kg/day

3 6 10

Losartan

B

% Renal fibrosis

3 10 3 6 10 (mg/kg/day)

Telmisartan Losartan

†††

C

(a) Telmisartan 3 mg/kg/day
(b) Telmisartan 10 mg/kg/day

3 10

Telmisartan

(c) Losartan 3 mg/kg/day
(d) Losartan 6 mg/kg/day
(e) Losartan 10 mg/kg/day

3 6 10

Losartan

D

% Positive area for macrophages (F4/80)

3 10 3 6 10 (mg/kg/day)

Telmisartan Losartan

††††
Figure S5.

A

(a) (b) (c) (d) (e)

Telmisartan

3 10 (mg/kg/day)

Losartan

3 6 10 (mg/kg/day)

B

Glomerular injury index

0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6

Telmisartan

3 10 (mg/kg/day)

Losartan

3 6 10 (mg/kg/day)

D

Tubulointerstitial injury score

0 0.5 1 1.5 2 2.5 3 3.5

Telmisartan

3 10 (mg/kg/day)

Losartan

3 6 10 (mg/kg/day)
Figure S5.
Glomerular injury index and tubulointerstitial injury score at each dosage of telmisartan and losartan
Histological analyses of glomerular injury and tubulointerstitial injury by semiquantitative morphometric evaluation.

A, Typical micrographs of renal glomerular sections with acid-Schiff staining to evaluate mesangial proliferation; (a) UUO-operated mice treated with telmisartan (3 mg/kg/day), (b) UUO-operated mice treated with telmisartan (10 mg/kg/day), (c) UUO-operated mice treated with losartan (3 mg/kg/day), (d) UUO-operated mice treated with losartan (6 mg/kg/day), (e) UUO-operated mice treated with losartan (10 mg/kg/day). Bar=50 μm.

B, Summary of glomerular injury index.
†P<0.05 vs. mice treated with telmisartan (3 mg/kg/day). Data are shown as mean ±SEM (n=5).

C, Typical micrographs of renal tubulointerstitial sections with acid-Schiff staining. Tubulointerstitial injury including macrophage infiltration and increased interstitial space as a result of ECM deposition; (a) UUO-operated mice treated with telmisartan (3 mg/kg/day), (b) UUO-operated mice treated with telmisartan (10 mg/kg/day), (c) UUO-operated mice treated with losartan (3 mg/kg/day), (d) UUO-operated mice treated with losartan (6 mg/kg/day), (e) UUO-operated mice treated with losartan (10 mg/kg/day). Bar=100 μm.

D, Summary of tubulointerstitial injury score.
†P<0.05 vs. mice treated with telmisartan (3 mg/kg/day). Data are shown as mean ±SEM (n=5).
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<td>IL-6</td>
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<td>CACGATTCCCAGAGAACATGTG</td>
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<tr>
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<tr>
<td>Sham</td>
<td>23.53 (±0.45)</td>
<td>82.5/53.6 (±3.1/4.3)</td>
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<tr>
<td>(-)</td>
<td>23.78 (±0.38)</td>
<td>83.6/55.7 (±4.1/5.1)</td>
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<tr>
<td>Losartan (3 mg/kg/day)</td>
<td>23.98 (±0.31)</td>
<td>83.5/52.8 (±3.8/2.1)</td>
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<td>Losartan (6 mg/kg/day)</td>
<td>23.31 (±0.53)</td>
<td>82.2/54.0 (±1.8/1.7)</td>
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<tr>
<td>Losartan (10 mg/kg/day)</td>
<td>22.47 (±0.69)</td>
<td>81.0/53.5 (±1.7/2.7)</td>
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<tr>
<td>Telmisartan (3 mg/kg/day)</td>
<td>23.56 (±0.64)</td>
<td>83.7/56.4 (±4.1/5.1)</td>
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<tr>
<td>Telmisartan (10 mg/kg/day)</td>
<td>23.10 (±0.89)</td>
<td>82.3/53.3 (±3.5/1.6)</td>
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<tr>
<td>Telmisartan (3 mg/kg/day) + HGF Ab</td>
<td>23.00 (±0.41)</td>
<td>84.9/53.8 (±4.3/3.7)</td>
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<tr>
<td>Telmisartan (3 mg/kg/day) + GW9662</td>
<td>21.78 (±0.76)</td>
<td>83.8/54.9 (±3.9/1.6)</td>
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Data are mean ± SEM
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<th>Groups</th>
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<th>Creatinine (mg/dl)</th>
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<td>0.17 (±0.01)</td>
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<tr>
<td>(−)</td>
<td>54.5 (±5.9)</td>
<td>0.28 (±0.02)</td>
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<td>Losartan</td>
<td>53.6 (±8.7)</td>
<td>0.27 (±0.03)</td>
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<tr>
<td>Telmisartan</td>
<td>50.4 (±6.7)</td>
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<tr>
<td>Telmisartan + HGF Ab</td>
<td>50.2 (±5.8)</td>
<td>0.25 (±0.02)</td>
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<tr>
<td>Telmisartan + GW9662</td>
<td>48.3 (±4.9)</td>
<td>0.24 (±0.01)</td>
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Data are mean ± SEM