Sustained-release prostacyclin analog ONO-1301 ameliorates tubulointerstitial alterations in a mouse obstructive nephropathy model

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Am J Physiol Renal Physiol 302: F1616–F1629, 2012. First published March 12, 2012; doi:10.1152/ajprenal.00538.2011.—Tubulointerstitial injuries are crucial historical alterations that predict the deterioration of renal function in chronic kidney disease. ONO-1301, a novel sustained-release prostacyclin analog, accompanied by thromboxane synthase activity, exerts therapeutic effects on experimental pulmonary hypertension, lung fibrosis, cardiomyopathy, and myocardial ischemia, partly associated with the induction of hepatocyte growth factor (HGF). In the present study, we examined the therapeutic efficacies of ONO-1301 on tubulointerstitial alterations induced by unilateral ureteral obstruction (UUO). After inducing unilateral ureteral obstruction in C57/BL6J mice, a single injection of sustained-release ONO-1301 polymerized with poly (l-lactic-co-glycolic acid) sustained-release ONO-1301 (SR-ONO) significantly suppressed interstitial fibrosis, accumulation of types I and III collagen, increase in the number of interstitial fibroblast-specific protein-1 (FSP-1) cells, and interstitial infiltration of monocytes/macrophages (F4/80+) in the obstructed kidneys (OBK; day 7). Treatment with SR-ONO significantly suppressed the increase of the renal levels of profibrotic factor TGF-β and phosphorylation of Smad2/3, and elevated the renal levels of HGF in the OBK. In cultured mouse proximal tubular epithelial cells (mProx24), ONO-1301 significantly ameliorated the expression of fibroblast-specific protein-1 and α-smooth muscle actin as well as phosphorylation of Smad3 and increased the expression of zonula occludens-1 and E-cadherin in the presence of TGF-β1 as detected by immunoblot and immunocytochemistry, partly dependent on PGI2 receptor-mediated signaling. Administration of rabbit anti-HGF antibodies, but not the control IgG, partly reversed the suppressive effects of SR-ONO on tubulointerstitial injuries in the OBK. Taken together, our findings suggest the potential therapeutic efficacies of ONO-1301 in suppressing tubulointerstitial alterations partly mediated via inducing HGF, an antifibrotic factor countering TGF-β.

Unilateral ureteral obstruction; prostacyclin; TGF-β1; HGF; FSP-1

Tubulointerstitial alterations are involved in the progression of chronic kidney disease (CKD). The histological features are characterized by interstitial infiltration of mononuclear cells, accumulation of myofibroblasts, proliferation of interstitial fibroblasts, and accumulation of extracellular matrix proteins, ultimately leading to interstitial fibrosis (3). In many forms of glomerular disorders, deterioration of renal function correlates better with the degree of tubulointerstitial injury rather than that of glomerular alterations (40). Therefore, the development of novel therapeutic strategies to inhibit the progression of tubulointerstitial alterations of CKD patients is required.

Unilateral ureteral obstruction (UUO) is a well-established experimental model of progressive renal tubulointerstitial injuries. Previous reports have shown the involvement of various mechanisms in chronic renal tubulointerstitial injuries in a UUO model including profibrotic TGF-β1 (20), and inflammatory cytokines, and chemokines such as IL-6 or monocyte chemoattractant protein-1 (51). More recently, the involvement of epithelial-mesenchymal transition (EMT) in the development of tubulointerstitial fibrosis has been demonstrated (13, 24, 64). In the process of EMT, epithelial cells dedifferentiate and lose epithelial cell surface markers accompanied by the acquisition of mesenchymal marker proteins, resulting in the formation of fibroblasts (53). The possibility of EMT as a contributor to renal interstitial fibrosis including UUO (4), as well as the involvement of various factors including TGF-β1 in the process of EMT and interstitial fibrosis, has also been reported (53).

Hepatocyte growth factor (HGF) possesses mitogenic, motogenic, and morphogenic activities in various cell types including tubular epithelial cells (22, 28). HGF is a potent antifibrotic and regenerative factor that prevents the progression of CKD in various experimental models including UUO (30), diabetic nephropathy (31), subtotal nephrectomy (12), ICR-derived glomerulonephritis mice (29), and adriamycin-induced podocyte injury (2). In addition, the biological effects of HGF to counteract TGF-β1 and EMT play important roles in attenuating renal fibrosis (12, 62).

Prostacyclin (PGI2), a metabolite of arachidonic acid, possesses vasoprotective effects including vasodilation, inhibition of platelet aggregation, and the proliferation of vascular smooth muscle cells (32, 33). In contrast, thromboxane (TX)A2 exerts the opposite effects to PGI2, contributing to vascular lesions (33). Currently, there are two known receptors mediating the biological effects of PGI2, the PGI2 receptor (IP) on the cell surface, and the nuclear peroxisome proliferator-activated receptor-δ. Prostacyclin-deficient mice by genetic dis-
ruption of PG12 synthetase developed renal fibrosis and arterial sclerosis (63), suggesting its important role in renal development and maintenance of a normal structure. Previous reports have demonstrated the renoprotective effects of beraprost sodium, a prostacyclin derivative, in various experimental models of renal disorders (19, 49, 58), including a rat partial UUO (55) model.

ONO-1301 is a synthetic nonprostanoid IP agonist possessing a potent inhibitory activity against thromboxane A2 (TXA2) synthase (6, 10, 34, 46). Although administration of prostacyclin generally leads to elevated plasma TXB2 levels, administration of ONO-1301 showed inhibitory effects on the increase of TXB2 in the rat pulmonary hypertension model (15), suggesting unique characteristics distinct from known prostacyclin derivatives. ONO-1301 specifically interacts with the IP receptor, but not with other types of prostaglandin receptors such as EP, TP, or FP receptors (18). Prostacyclin and its analogs are not stable in vivo, since 15-hydroxy PG dehydrogenase metabolizes their prostanoid structures. Unlike prostacyclin, ONO-1301 lacks the typical prostanoid structures, including a five-member ring and allylic alcohol, thus leading to the improved biological and chemical stability of this compound in vivo.

The therapeutic effects of ONO-1301 in experimental models of pulmonary fibrosis (34), pulmonary hypertension (15), and ischemic heart disease (37) have been reported. The beneficial effect of ONO-1301 in treating cardiac ischemia was partly mediated via upregulation of HGF and VEGF, via a cAMP-dependent pathway (37). More recently, a novel sustained-release ONO-1301 (SR-ONO) has been developed. A single subcutaneous injection of SR-ONO resulted in sustained elevation of its circulating levels for 3 wk (41).

A previous report has demonstrated the antinephritic effects of ONO-1301 (twice daily) in a rat anti-GBM nephritis model (6). More recently, we reported the therapeutic effects of SR-ONO in a rat model of type 1 diabetic nephropathy (60). However, the therapeutic effects of ONO-1301 on tubulointerstitial injuries have not been demonstrated to date.

In the present study, we hypothesized that ONO-1301 may ameliorate tubulointerstitial injuries in a mouse UUO model through the antifibrotic effects mediated partly via the induction of HGF blockade of EMT, and downregulation of TGF-β1 signaling.

MATERIALS AND METHODS

Preparation of SR-ONO. ONO-1301 was synthesized by ONO Pharmaceutical, (Osaka, Japan) as previously described (6, 10, 34). A slow-release form of ONO-1301 (SR-ONO) was generated by encapsulating ONO-1301 with poly (d,l-lactic-co-glycolic acid) (PLGA) as described previously (14, 41). (See APPENDIX.)

Experimental protocol. The experimental protocol was approved by the Animal Ethics Review Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan. Studies were performed in male C57BL/6 mice. The animals were fed a standard pellet laboratory diet and were provided with water ad libitum. With the mice under pentobarbital anesthesia, the left ureter was exposed through a midabdominal incision and ligated with 3-0 silk at two locations ~1 cm below the renal hilum, as described previously (50, 54). Sham-operated mice had their ureters manipulated but not ligated. Weight-matched (20–22 g) 10-wk-old mice were divided into four subgroups: 1) undiseased, 2) vehicle (PLGA polymer containing no active substance)-treated UUO, 3) SR-ONO (3 mg/kg body wt)-treated UUO, 4) SR-ONO (10 mg/kg body wt)-treated UUO (n = 5 for each subgroup). Mice received subcutaneous injection of SR-ONO or vehicle buffer on the day of initiation of UUO. Mice were killed on day 7, and the bilateral kidneys and the serum were obtained.

For blockade of endogenous HGF, neutralizing rabbit anti-rat/mouse HGF antibody (30) was used. Rat recombinant HGF was purified from the culture medium of Chinese hamster ovary cells transfected with plasmid for expression of rat HGF (57). Polyclonal antibody against rat HGF was raised in rabbits by immunizing with rat recombinant HGF (42). IgG was purified using protein-A Sepharose (Sigma Aldrich, St. Louis, MO). Anti-rat HGF IgG at 3 μg/ml almost completely neutralizes the biological activity of rat and mouse HGF at 10 ng/ml. The dose and interval for administration of anti-rat HGF IgG in mice were determined by another experiment to examine the inhibitory effects of anti-rat/mouse HGF IgG on liver regeneration in mice (data not shown). Male C57BL/6 mice (5 wk old) receiving UUO were subcutaneously injected with SR-ONO (10 mg/kg body wt) or vehicle buffer once on the day of initiation of UUO. The SR-ONO-treated UUO mice were randomly divided into two groups and received intraperitoneal injections of either rabbit anti-rat HGF IgG (n = 5) or normal rabbit IgG (n = 5) every other day (250 μg/mouse). Mice were killed on day 7 and the bilateral kidneys and the serum were obtained. No mice died and no signs of apparent exhaustion were observed during the experimental period.

Blood examination. Blood urea nitrogen (BUN) and serum creatinine levels were measured at SRL (Okayama, Japan). Serum creatinine levels were measured by the enzymatic colorimetric method as described (5). The serum ONO-1301 level was measured by liquid chromatography tandem mass spectrometry assay (41).

Histological analysis. At 7 days after the induction of UUO, kidneys were removed, fixed in 10% buffered formalin, and embedded in paraffin. Sections (4 μm thick) were stained with Masson’s Trichrome for light microscopic observation. Blue-stained interstitial fibrotic areas were assessed by computer-image analysis using Luminia Vision software (Mitani, Fukui, Japan). Fifteen consecutive fields were randomly selected in the renal cortex and evaluated at magnification ×400. Tubular cells, tubular lumen, glomeruli, or blood vessels were not included. This fraction represented the relative interstitial volume. Results were expressed as a percentage of the measured area, which represented the interstitial space and was determined as the relative volume of the interstitium (50). Histological evaluation was performed in a blinded fashion by two investigators and averaged.

Immunohistochemistry. Immunohistochemistry was performed using 4-μm frozen sections as described previously (8, 26, 39, 56). Briefly, frozen kidney sections were incubated with polyclonal rabbit anti-human fibroblast-specific protein-1 (FSP-1; S100A4) antibody (1:100 dilution, AS114; Dakocytomation, Carpinteria, CA) and rat anti-mouse F4/80 antibody (1:200 dilution, A3–1; Serotec, Oxford, UK) and exposed to secondary antibody, Immun-Star goat anti-rabbit (GAR)-horseradish peroxidase (HRP) conjugate (1:200 dilution, 170–5046; Bio-Rad, Hercules, CA), and HRP-labeled goat anti-rat IgG (Millipore, Billerica, MA). Diaminobenzidine was used as a chromogen. All slides were counterstained with hematoxylin. Renal interstitial monocyte/macrophage infiltration was determined by counting the number of F4/80+ cells in the renal cortex. The number of F4/80+ cells was determined in 15 randomly selected nonoverlapping fields (magnification, ×400) in each section of the individual mouse renal cortex. The average number of F4/80+ cells from five separate animals was calculated and averaged (50). Similarly, the number of FSP-1+ cells in the tubulointerstitium was determined (magnification, ×400). For immunohistochemistry of mouse IP receptors, formalin (10%)-fixed, paraffin-embedded sections (4 μm) were used. After deparaffinization, sections were incubated with rabbit polyclonal anti-IP receptor antibody (1:100 dilution; Cayman Chemical, Ann Arbor, MI) followed by incubation with biotinylated-secondary anti-
body, and immunoperoxidase staining was carried out. Diaminobenzidine was used as a chromogen. All slides were counterstained with hematoxylin. (See APPENDIX.)

**Immunofluorescence.** Immunofluorescent staining was performed as described previously (9, 39, 56) to assess interstitial accumulation of types I and III collagen. (See APPENDIX.)

**Western blot analysis.** Western blot was performed as described previously (9, 27, 39, 47, 56) to assess renal levels of TGF-β, pSmad3, and HGF. (See APPENDIX.)

**Cell culture.** Immortalized murine proximal tubular cells (mProx24) derived from microdissected proximal tubular segments of C57BL/6J adult mouse kidney were used as previously described (16). The mProx24 cells were cultured in DMEM (high glucose 4500 mg) culture medium (Sigma Aldrich) supplemented with 10% FBS, penicillin G (100 U/ml), and 100 μg/ml of streptomycin. The mProx24 cells were trypsinized and a suspension of 3 × 10⁶ cells in DMEM with 10% FBS added to each well of a six-well plate precoated with 1% gelatin. The cells were incubated for 24 h at 37°C, and after 80% confluence, the medium was replaced with DMEM supplemented with 0.2% FBS and incubated for 24 h. The cells were incubated with 0.2% FBS media containing ONO-1301 (0.1, 0.5, 1, 2.5, and 10 nM) and recombinant human TGF-β1 (0.1 nM, 240-B; R&D Systems, Minneapolis, MN) and incubated for 24 h. In another set of experiments, cells were preincubated with CAY10449 (1–10 μM), an IP receptor antagonist, for 30 min, prior to stimulation with recombinant human TGF-β1 (0.1 nM, 240-B; R&D Systems, Minneapolis, MN) and incubated for 24 h. In another set of experiments, cells were stimulated with IP receptor agonists (CAY10449) and incubated for 24 h. The cells were incubated with 0.2% FBS and incubated for 24 h. The cells were incubated with 0.2% FBS media containing ONO-1301 (0.1, 0.5, 1, 2.5, and 10 nM) and recombinant human TGF-β1 (0.1 nM, 240-B; R&D Systems, Minneapolis, MN) and incubated for 24 h. In another set of experiments, cells were preincubated with CAY10449 (1–10 μM), an IP receptor antagonist, for 30 min, prior to stimulation with recombinant human TGF-β1 (0.1 nM, 240-B; R&D Systems, Minneapolis, MN) and incubated for 24 h. In another set of experiments, cells were stimulated with IP receptor agonists (CAY10449) and incubated for 24 h.

They then were harvested and subjected to Western blot analysis to determine the effect of ONO-1301 on the protein levels of FSP-1, E-cadherin, phosphorylated Smad3, and total Smad3; anti-FSP-1 (S100A4) antibody (1:1000 dilution, A5114, Dakocytomation), monoclonal rat anti-E-cadherin antibody (1:500 dilution, U3254, Sigma Aldrich), rabbit anti-Smad3 antibody (1:1,000 dilution, C67H9, Cell Signaling Technology, Danvers, MA), rabbit anti-phospho-Smad3 (pSmad3; Ser423/425) antibody (1:1,000 dilution, 0.2% FBS and incubated for 24 h. The cells were incubated with 0.2% FBS media containing ONO-1301 (0.1, 0.5, 1, 2.5, and 10 nM) and recombinant human TGF-β1 (0.1 nM, 240-B; R&D Systems, Minneapolis, MN) and incubated for 24 h. In another set of experiments, cells were preincubated with CAY10449 (1–10 μM), an IP receptor antagonist, for 30 min, prior to stimulation with recombinant human TGF-β1 (0.1 nM, 240-B; R&D Systems, Minneapolis, MN) and incubated for 24 h. In another set of experiments, cells were stimulated with IP receptor agonists (CAY10449) and incubated for 24 h. The cells were incubated with 0.2% FBS and incubated for 24 h. The cells were incubated with 0.2% FBS media containing ONO-1301 (0.1, 0.5, 1, 2.5, and 10 nM) and recombinant human TGF-β1 (0.1 nM, 240-B; R&D Systems, Minneapolis, MN) and incubated for 24 h. In another set of experiments, cells were preincubated with CAY10449 (1–10 μM), an IP receptor antagonist, for 30 min, prior to stimulation with recombinant human TGF-β1 (0.1 nM, 240-B; R&D Systems, Minneapolis, MN) and incubated for 24 h.

Expression and localization of mouse IP receptors in kidney. mRNA for IP receptors was observed in the mouse kidney (normal control) and in mProx24, proximal tubular epithelial cells (Fig. 1B). Immunoreactivity for IP receptors was observed in the proximal and distal tubules and in the smooth muscle cells of interlobular arteries in the kidney cortex of normal mice (Fig. 1, C and E). In the kidneys of the control UUO mice, immunoreactivity for IP receptors was observed in the dilated tubules (Fig. 1D).

**Histology and morphometric analysis.** Morphological alterations of kidney tissue were analyzed by Masson’s Trichrome staining. Tubular dilation, atrophy, interstitial infiltration of inflammatory mononuclear cells and interstitial fibrosis are the characteristic histological alterations observed in obstructive nephropathy. These alterations were observed in the OBK on day 7 after inducing UUO in the vehicle-treated group (Fig. 2B). Treatment with SR-ONO at the dosage of 3 mg/kg (Fig. 2C) and 10 mg/kg (Fig. 2D) markedly ameliorated these histological alterations in the OBK on day 7. Interstitial fibrosis in the OBK was significantly increased in the vehicle-treated UUO group compared with the sham-operated control group (Fig. 2E). SR-ONO treatment at the dosage of 3 or 10 mg/kg, significantly suppressed the increase of the interstitial fibrotic area in the OBK on day 7 after initiating UUO compared with the vehicle-treated UUO group (Fig. 2E). There were no evident morphological differences in the glomeruli or intrarenal blood vessels in the OBK of any experimental group. No significant histological alterations were observed in the tubulointerstitial of the sham-operated control group or contralateral kidneys (data not shown) by light microscopic observation.

**Immunohistochemical analysis of interstitial types I and III collagens.** We next examined the cortical interstitial accumulation of type I and III collagens in association with interstitial fibrosis in the OBK. Immunoreactivity for interstitial type I and III collagens was increased in the OBK of the vehicle-treated UUO group (Fig. 3, B and F) on day 7 after inducing UUO compared with the undiseased control group (Fig. 3, A and E). Treatment with SR-ONO at the dosage of 3 mg/kg (Fig. 3, C and G) and 10 mg/kg (Fig. 3, D and H) ameliorated the accumulation of these interstitial collagens in the OBK on day 7. Computer image analysis confirmed that treatment with SR-ONO either at the lower or higher dosage significantly inhibited interstitial accumulation of type I and III collagens in the OBK (Fig. 3, I and J).

**Immunohistochemical analysis of interstitial FSP-1⁺ cells.** Renal interstitial fibrosis is associated with the accumulation of a single subcutaneous injection of SR-ONO. Serum levels of ONO-1301 were significantly elevated in the SR-ONO-treated group, whereas ONO-1301 was below the detectable limit in the undiseased control and vehicle-treated UUO group (Fig. 1). These results demonstrate that a single subcutaneous administration of SR-ONO maintains effective circulating levels of ONO-1301 for 7 days.

**Body weight, kidney weight, and renal function.** Body weight was not significantly different among experimental groups. The left obstructed kidney (OBK) weight of vehicle-treated UUO mice on day 7 was significantly greater compared with the sham-operated control. Serum creatinine and blood urea nitrogen levels were not significantly different among the experimental groups (Table 1).

All values are expressed as means ± SE. A Kruskal-Wallis test with post hoc multiple comparisons using Scheffé’s test was employed for intergroup comparisons of multiple variables. A level of P < 0.05 was considered statistically significant.

**RESULTS**

**Serum levels of ONO-1301.** A previous report has demonstrated that subcutaneous injection of SR-ONO resulted in stable circulating levels of ONO-1301 for 3 wk (41). To investigate the pharmacokinetics in vivo in the present model, we measured serum concentrations of ONO-1301 at day 7 after a single subcutaneous injection of SR-ONO. Serum levels of ONO-1301 were significantly elevated in the SR-ONO-treated group, whereas ONO-1301 was below the detectable limit in the undiseased control and vehicle-treated UUO group (Fig. 1). These results demonstrate that a single subcutaneous administration of SR-ONO maintains effective circulating levels of ONO-1301 for 7 days.
fibroblasts and their contractile subtype, the myofibroblasts. We next examined interstitial accumulation of fibroblasts in the OBK by immunohistochemistry for FSP-1. Although very small numbers of interstitial FSP-1+ fibroblasts were observed in the undiseased control group (Fig. 4A), marked interstitial accumulation of fibroblasts was observed in the OBK of the vehicle-treated animals (Fig. 4B) on day 7 after inducing UUO. Treatment with SR-ONO at the dosage of 3 mg/kg significantly ameliorated interstitial accumulation of FSP-1+ fibroblasts in the OBK (Fig. 4C), and SR-ONO administration at the dosage of 10 mg/kg further reduced accumulation of fibroblasts (Fig. 4D). Quantitative analysis confirmed the significant inhibitory effects of SR-ONO (Fig. 4I) on interstitial fibroblast accumulation. No apparent glomerular infiltration of FSP-1+ fibroblasts was observed in any of the experimental groups.

Immunohistochemical analysis of interstitial infiltration of F4/80+ monocytes/macrophages. We next examined interstitial infiltration of monocytes/macrophages in the OBK, a hallmark of tubulointerstitial injuries, by immunohistochemistry for F4/80. Although a very small number of interstitial F4/80+ monocytes/macrophages were observed in the undiseased control group (Fig. 4E), marked interstitial accumulation was observed in the OBK of the vehicle-treated UUO group (Fig. 4F) on day 7 after inducing UUO. Treatment with SR-ONO at the dosage of 3 mg/kg ameliorated interstitial infiltration of monocytes/macrophages in the OBK (Fig. 4G), and SR-ONO administration at the dosage of 10 mg/kg (Fig. 4H) further reduced monocyte/macrophage infiltration. Quantitative analysis confirmed the significant inhibitory effects of SR-ONO on interstitial monocyte/macrophage infiltration (Fig. 4J). No apparent glomerular infiltration of F4/80+ monocytes/macrophages was observed in any of the experimental groups.

Protein levels of TGF-β and activation of Smad2/3. We next examined the potential effect of SR-ONO on regulating the levels of profibrotic TGF-β and the activation of its downstream effector molecule Smad2/3. TGF-β1 serves as a profibrotic growth factor involved in the accumulation of glomerular and interstitial matrix proteins in various renal disorders. Increase in the protein levels of TGF-β in the OBK was observed at day 7 after initiating UUO compared with the undiseased control group (Fig. 5A). Treatment with SR-ONO at the dosage of 3 mg/kg resulted in the significant suppression of TGF-β levels compared with the vehicle-treated UUO group and further suppressed at the dosage of 10 mg/kg (Fig. 5A).

Table 1. Body weight, kidney weight, and renal function

<table>
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<th>Control</th>
<th>Vehicle</th>
<th>ONO-1301, 3 mg</th>
<th>ONO-1301, 10 mg</th>
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<tr>
<td>Body weight, g</td>
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<td>19.8 ± 0.27</td>
<td>20.3 ± 0.15</td>
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<td>Left kidney, mg</td>
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<td>136.7 ± 5.3*</td>
<td>133.3 ± 12.5</td>
<td>123.3 ± 8.6</td>
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<tr>
<td>Right kidney, mg</td>
<td>125.0 ± 5.0</td>
<td>133.2 ± 3.9</td>
<td>133.3 ± 9.6</td>
<td>143.3 ± 13.7</td>
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<tr>
<td>BUN, mg/dl</td>
<td>33.3 ± 3.0</td>
<td>40.4 ± 3.5</td>
<td>39.7 ± 3.6</td>
<td>34.5 ± 1.0</td>
</tr>
<tr>
<td>sCr, mg/dl</td>
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<td>0.23 ± 0.01</td>
<td>0.23 ± 0.01</td>
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Values are means ± SE. BUN, blood urea nitrogen; sCr, serum creatinine. *P < 0.05 vs. control.

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The Smad family comprises several members, and TGF-β induces phosphorylation of Smad2 and Smad3. Phosphorylated Smad2/3 interacts with Smad4, translocates into the nucleus, and modulates transcriptional activation of genes regulated by TGF-β. We next examined the activation of Smad2/3 in the experimental groups. Levels of phosphorylated Smad2/3 in the renal cortex of the OBK were significantly increased at day 7 after initiating UUO compared with the undiseased control group (Fig. 5B). Treatment with SR-ONO at the dosage of 10 mg/kg resulted in significant suppression of phosphorylated Smad2/3 levels compared with the vehicle-treated UUO group (Fig. 5B).

Renal protein levels of HGF. We next examined the potential effect of SR-ONO treatment on the renal levels of HGF, a factor counteracting the profibrotic effects of TGF-β. The protein levels of HGF in the renal cortex of the OBK (day 7) in the vehicle-treated group were not significantly different compared with the undiseased control group (Fig. 6). Treatment with SR-ONO at the dosage of 3 or 10 mg/kg resulted in significant elevation of the HGF levels in the OBK compared with the vehicle-treated UUO group (Fig. 6). These results suggest that ONO-1301 may ameliorate tubulointerstitial injuries in the present model via induction of HGF, potentially counteracting the profibrotic effects TGF-β1.

Immunohistochemical analysis of cultured mProx24 cells. We next evaluated the direct effect of ONO-1301 to protect the phenotype of tubular epithelial cells by immunohistochemistry for FSP-1, α-SMA, ZO-1, and E-cadherin using mProx24 cells. Light microscopic appearance of mProx24 cells under the basal condition in the absence (Fig. 7A) or presence of ONO-1301 (10 nM; Fig. 7B) was cobblestone-like, and immunocytochemistry confirmed the epithelial phenotype with low levels of mesenchymal markers, such as FSP-1 (Fig. 7, F and G), the absence of α-SMA (Fig. 7, K and L), and the expression of epithelial cell markers ZO-1 (Fig. 7, P and Q) and E-cadherin (Fig. 7, U and V). Incubation with TGF-β1 led to dramatically altered appearance of mProx24 cells exhibiting the spindle-like shape (Fig. 7C), accompanied by the increase of FSP-1 (Fig. 7H) and the expression of α-SMA (Fig. 7M) as well as the reduction of ZO-1 (Fig. 7K) and E-cadherin (Fig. 7W), suggesting EMT. Treatment with ONO-1301 reversed these EMT-like alterations induced by TGF-β1 toward reacquisition of the epithelial phenotype (Fig. 7, D, I, N, S, and X). Treatment with CAY10449 (1 μM), an IP receptor antagonist, partly reversed the beneficial effect of ONO-1301 (Fig. 7, E, J, O, T and Y).

Protein levels of FSP-1, E-cadherin, and pSmad3 in cultured mProx24 cells. The regulatory effects of ONO-1301 on the protein levels of FSP-1, E-cadherin, and pSmad3 in mProx24 cells were further confirmed by the immunoblot. The protein levels of FSP-1 were significantly increased at 24 h in the presence of TGF-β1. Treatment with ONO-1301 significantly suppressed the TGF-β1-induced increase of the protein levels of FSP-1 in a dose-dependent manner (Fig. 8A). Protein levels of E-cadherin in mProx24 cells were slightly decreased at 24 h in the presence of TGF-β1, and treatment with ONO-1301 significantly increased the levels of E-cadherin (Fig. 8B). Treatment with CAY10449 (1 μM) significantly increased the protein levels of FSP-1 (Fig. 8C) and suppressed the levels of E-cadherin (Fig. 8D) in the presence of ONO-1301 and TGF-β1. These data suggest that ONO-1301 may ameliorate TGF-β1-induced EMT of mProx24 cells, partly through IP receptor-mediated signaling. Increase in the levels of pSmad3 was significantly ameliorated by treatment with ONO-1301, but addition of CAY10449 did not reverse the effect of ONO-1301 (Fig. 8E).

Administration of the neutralizing anti-HGF antibodies reversed the therapeutic effects of ONO-1301. We next examined the involvement of HGF in the therapeutic effects of ONP-1301 on tubulointerstitial injuries by administrating neutralizing anti-HGF antibodies. Administration of the control IgG (Fig. 9, C, G, K, and O) did not affect the suppressive effects by treatment with ONO-1301 (Fig. 9, B, F, J, and N) on interstitial fibrosis, accumulation of interstitial collagens, and the interstitial FSP-1+ fibroblasts in the OBK. However, administration of the neutralizing anti-HGF antibodies significantly reversed the observed therapeutic effects of ONO-1301.
in the OBK (Fig. 9, D, H, L, and P–T). These results suggest that ONO-1301 may ameliorate tubulointerstitial injuries in a mouse UUO model, at least in part, through induction of HGF in association with its antifibrotic effects.

**DISCUSSION**

The induction of UUO with acute and complete obstruction of the ureter is followed by inflammatory cell infiltration, proliferation and apoptosis of tubular epithelial cells, tubular...
atrophy, accumulation of myofibroblasts and fibroblasts, and interstitial fibrosis (1), implicating that this experimental model is suitable for investigating a novel therapeutic approach to ameliorate tubulointerstitial alterations.

Previous reports have demonstrated the therapeutic effects of prostacyclin and its derivatives in the experimental model of renal disorders. PGE1 and the inhibitor of TXA2 synthase ameliorated experimental glomerulonephritis through vasodilative and anticoagulative effects (35, 36). Continuous infusion of beraprost sodium, a prostacyclin analog, resulted in the amelioration of rat crescentic glomerulonephritis (19) and of experimental diabetic nephropathy (58, 61). More recently, twice-daily oral administration of beraprost sodium suppressed renal alterations in an obesity-induced type 2 diabetes model (49) and in a rat partial UUO model (55). However, the mechanism underlying the therapeutic effects of prostacyclin analog in renal tubulointerstitial alterations largely remains unknown. The TX receptor antagonist S18886 exhibited renoprotective effects through attenuating renal oxidative stress in diabetic apolipoprotein E-deficient mice (59) and in uninephrectomized obese Zucker rats (52). Earlier studies reported that treatment with the TXA2 receptor antagonist resulted in reduction of renal vascular resistance and amelioration of histological alterations of early obstruction following relief of 24 h of UUO (45). However, the biological role of TXA2 in the context of chronic tubulointerstitial injuries still remains to be elucidated.

In the present study, we evaluated the therapeutic efficacies of ONO-1301, a novel prostacyclin analog also possessing inhibitory activity on TXA2 synthase, on renal tubulointerstitial alterations in a mouse UUO model. Although unique characteristics of ONO-1301 lacking prostanoid structures prolonged the half-life of the compound, twice daily administration of ONO-1301 was still necessary for maintaining therapeutic effects (15). Considering the chronic disease course of patients with CKD, development of a long-acting prostacyclin analog possessing therapeutic efficacies would be favored. Previous reports have shown that a single subcutaneous administration of SR-ONO achieved sustained elevation of its circulating levels for 3 to 4 wk (14, 41). In spite of sustained circulating levels of ONO-1301, desensitization of IP receptors is unlikely. Of note, the inhibitory effect of beraprost sodium on platelet aggregation gradually decreases following repeated administration, but that of ONO-1301 remains at a constant level in rabbits (Sakai Y, unpublished observations).

A single administration of SR-ONO at the dosage of 10 mg/kg body wt resulted in sufficient circulating levels of ONO-1301 at day 7 in the present study, within the reported effective therapeutic range (41, 60). Although serum levels of ONO-1301 in animals receiving SR-ONO at the dosage of 3 mg/kg were lower than the levels observed in our previous study (60), significant therapeutic effects on tubulointerstitial injury were observed. Our results may suggest that serum levels of ONO-1301 at 1 ng/ml would be sufficient to expect therapeutic effects on tubulointerstitial injuries in the present model. Successful therapeutic effects of SR-ONO on renal tubulointerstitial injuries achieved by a single administration implicate its potential to serve as a novel therapeutic strategy.
for CKD, compared with the other prostacyclin derivatives requiring more frequent administration.

The dosage of SR-ONO in the present study was determined based on previous studies (34, 60) and preliminary experimental results (Sakai Y, unpublished observations). A previous report has demonstrated that the administration of SR-ONO at the dosage of 300 mg/kg led to significant hypotension, but no such systemic effects were observed at 100 mg/kg in rodents (41). In the present study, we observed no adverse reactions, such as apparent flushing of skin, diarrhea, or liver dysfunction by treatment with SR-ONO. Thus, SR-ONO appears to enable ONO-1301 to be delivered continuously and safely in chronic disorders such as CKD. For clinical use, it would be feasible to optimize the size of microspheres, the timing of release, and the content of ONO-1301.

SR-ONO treatment significantly attenuated interstitial accumulation of monocyte/macrophages in the OBK, consistent with the findings of previous reports (60, 61). Yamashita et al. (61) demonstrated that the anti-inflammatory action of prost sodium might be associated with downregulation of glomerular intercellular adhesion molecule-1 (ICAM-1) levels in the experimental diabetic nephropathy. The anti-inflammatory effects of SR-ONO in association with the suppressive effects on ICAM-1 levels have also been reported in experimental pulmonary fibrosis (34). Since macrophages secrete TGF-β (21), the observed anti-inflammatory effects of SR-ONO might have partly contributed to the antifibrotic effects in the present model.

The inhibitory effects of SR-ONO on the accumulation of interstitial collagens in the OBK are consistent with the reported antifibrotic effects in the experimental pulmonary fibrosis (34) and diabetic nephropathy (58, 60) models. Previous reports have demonstrated Smad3, a key signaling intermediate downstream of the TGF-β receptors, as a key molecule mediating TGF-β-induced renal fibrosis in the UUO model (11, 48). Treatment with SR-ONO reduced interstitial fibrosis, interstitial accumulation of fibroblasts (FSP1) in the OBK in parallel with the suppressive effects on the levels of TGF-β, and

Fig. 7. The morphology and immunofluorescent staining for FSP-1, α-SMA, zonula occludens-1 (ZO-1), and E-cadherin using cultured mProx24. Light microscopic appearance (A–E) and immunofluorescent staining of FSP-1 (F–J), α-SMA (K–O), ZO-1 (P–T), and E-cadherin (U–Y) are shown. Distribution of FSP-1 (green), α-SMA (red), ZO-1 (green), and E-cadherin (red) in mProx24 cells was examined by indirect immunofluorescent staining, cultured in the presence or absence of TGF-β (0.1 nM), ONO-1301 (10 nM), and CAY10449 (1 µM). Cells were also double stained with 4′, 6-diamino-2-phenylindole HCL to visualize the nuclei. A, F, K, P and U: unstimulated basal condition. B, G, L, Q and V: mProx24 cells were incubated with ONO-1301 in the absence of TGF-β. C, H, M, R and W: mProx24 cells were preincubated with CAY10449 (1 µM) and then stimulated by TGF-β in the presence of ONO-1301. E, J, O, T and Y: mProx24 cells were preincubated with CAY10449 (1 µM) and then stimulated by TGF-β in the presence of ONO-1301. Original magnification, × 400.
phosphorylation of Smad3. Of note, treatment with ONO-1301 significantly suppressed TGF-β1-induced phosphorylation of Smad3 in mProx24 cells. These results suggest the involvement of regulatory mechanisms on TGF-β signaling in mediating the antifibrotic effects of SR-ONO in the present model.

Previous studies have demonstrated the upregulation of HGF induced by ONO-1301 in experimental ischemic heart disease models (14, 37). HGF inhibits the expression of TGF-β1, counters the action of TGF-β1, and antagonizes TGF-β1-induced EMT, tubular epithelial cell apoptosis, interstitial fibroblast activation, and endothelial cell apoptosis of peritubular capillaries (30, 62). In the present study, we observed significant upregulation of renal HGF levels in the OBK following treatment with SR-ONO, consistent with studies using ischemic heart disease models (14, 37). These results clearly implicate the significant involvement of HGF induced by ONO-1301 in ameliorating tubulointerstitial injuries in the present model. Although not examined in the present study, evaluation on the levels and activation of MET, a tyrosine kinase receptor for HGF, following treatment with ONO-1301 requires further investigation.

EMT is the process by which fully differentiated epithelial cells undergo transition that gives rise to the matrix-producing fibroblasts and myofibroblasts and has been recognized as one of the key mechanisms involved in renal fibrosis (23, 64). The transition is characterized by the loss of epithelial proteins such as E-cadherin, ZO-1, and cytokeratin, and acquisition of the expression of the mesenchymal markers including vimentin, α-smooth muscle actin (α-SMA), and FSP-1 (23). In the present study, the phenotypic conversion of the cultured mouse proximal tubular epithelial cells induced by TGF-β1 was

Fig. 8. Immunoblot analysis (mProx24 cells). A–D: immunoblots for FSP-1, E-cadherin (E-Cad) and actin are shown. A, bottom: intensities of FSP-1 protein relative to actin are shown. *P < 0.05 vs. unstimulated groups. †P < 0.05 vs. TGF-β1-stimulated, ONO-1301-untreated groups. B, bottom: intensities of E-cadherin protein relative to actin are shown. *P < 0.05 vs. unstimulated groups. †P < 0.05 vs. TGF-β1-stimulated, ONO-1301-untreated groups. C, bottom: intensities of FSP-1 protein relative to actin are shown. *P < 0.05 vs. CAY10449-untreated groups. D, bottom: intensities of E-cadherin protein relative to actin are shown. *P < 0.05 vs. TGF-β1-stimulated, ONO-1301-untreated groups. ONO-1301-untreated groups. CAY10449-untreated groups. E: immunoblots for pSmad3 and total Smad3 are shown. A, bottom: intensities of pSmad3 protein relative to total Smad3 are shown. B, bottom: intensities of pSmad3 protein relative to total Smad3 are shown. C, bottom: intensities of FSP-1 protein relative to actin are shown.
observed. Treatment with ONO-1301 in vitro potently down-regulated TGF-β1-induced upregulation of mesenchymal markers and recovered the expression of epithelial markers, suggesting its role of ameliorating EMT in the context of UUO. Interestingly, treatment with neutralizing anti-HGF antibodies failed to affect the regulatory role of ONO-1301 on TGF-β1-induced EMT in vitro (data not shown), suggesting that distinct cell-types other than tubular epithelial cells might have produced HGF in response to ONO-1301 in vivo, hence leading to the therapeutic effects in the UUO model. The paradigm of EMT in the involvement of renal fibrosis has recently been challenged based on the findings of the new fate-mapping or lineage-tracing experiments, in which the epithelium is genetically tagged, enabling the identification of cells of epithelial

**Fig. 9.** Administration of the neutralizing anti-HGF antibodies reversed the antifibrotic effects of ONO-1301. Representative photomicrographs of the renal cortex of OBK (A–D; Masson’s Trichrome staining), immunohistochemistry of FSP-1 (E–H; arrowheads, FSP-1 fibroblasts), and immunofluorescent analysis of types I (I–L) and III (M–P) collagen for vehicle-treated UUO animals (A, E, I, and M), UUO animals treated with SR-ONO at the dosage of 10 mg/kg (B, F, J, and N), UUO animals treated with SR-ONO and control IgG (C, G, K, and O), and UUO animals treated with SR-ONO and anti-HGF antibodies (D, H, L, and P) are shown (day 7). Original magnification, ×400. Q: increase of interstitial fibrotic areas. R: number of interstitial FSP-1+ fibroblasts. S: accumulation of interstitial types I (S) and III (T) collagen (staining index ratio relative to the vehicle-treated UUO animals) are shown. *P < 0.01 vs. vehicle-treated UUO animals. †P < 0.05 vs. SR-ONO (10 mg)-treated UUO animals (n = 5 for each group). Each column consists of means ± SE.
origin throughout the disease process (7). The concept of fibrogenic EMT has proved to be vastly fruitful, and numerous publications provided observational and experimental support in regard to the contribution of EMT to tubulointerstitial fibrosis, as recently reviewed by Quaggin et al. (44). Although the involvement of EMT in renal fibrosis still remains controversial, the results of the present study suggest the regulatory role of ONO-1301 on EMT, potentially associated with its therapeutic effects in ameliorating tubulointerstitial injuries.

IP receptors are localized to platelets, vascular smooth muscle cells, vascular endothelial cells, and glomerular cells, namely mesangial cells, endothelial cells, and podocytes, and also in distal tubules and collecting ducts in the human kidney (17). In the rodent kidney, IP receptors were localized to the afferent arterioles, glomeruli (61), and to various cell types of nephron including proximal tubular epithelial cells (38, 43). In the present study, we observed mRNA as well as protein expression of IP receptors in the proximal tubular epithelial cells. In addition, IP receptors were observed in the vessel walls of the interlobular arteries. Therefore, ONO-1301 might have exerted therapeutic effects via protective effects on tubular epithelial cells and blood vessels. The anti-inflammatory action of ONO-1301 may derive from reduced expression of ICAM-1 on endothelial cells and from inhibition of the production of cytokines/chemokines such as TNF-α and monocyte chemoattractant protein-1 in tubular epithelial cells. ONO-1301 may inhibit interstitial fibrosis via directly suppressing EMT of tubular epithelial cells as demonstrated by cell culture analysis, and via inducing HGF production in nontubular cells, indirectly suppressing the profibrotic effects of TGF-β on myofibroblasts and fibroblasts.

Since treatment with CAY10449 partly reversed the inhibitory effects of ONO-1301 on EMT in mProx24 cells, ONO-1301 may exert its renoprotective effects partly through IP receptors. Interestingly, treatment with CAY10449 failed to reverse the suppressive effects of ONO-1301 on phosphorylation of Smad3, suggesting the involvement of distinct pathway such as peroxisome proliferator-activated receptor-δ.

There are several limitations to the present study. Although not observed in the present study, systemic administration of SR-ONO may lead to unexpected adverse events such as hypotension, flushing, and diarrhea in humans, requiring further preclinical studies confirming the safety and efficacies of SR-ONO in patients with CKD. The present therapeutic effects of SR-ONO were observed by initiating therapy from the initiation of UUO, further requiring confirmation of therapeutic effects by beginning administration of SR-ONO at a later stage. Since we observed amelioration of interstitial fibrosis by treatment with SR-ONO, further assessment on its direct effect on fibroblasts in addition to tubular epithelial cells might be required. Since ONO-1301 exerts its effects mainly through IP receptors and also inhibits TXA2 synthase activity, the potential contribution of the latter capacity on the suppressive effects of SR-ONO, thus leading to the improved balance between prostacyclin and TBX, requires further clarification.

In conclusion, we demonstrated that SR-ONO effectively ameliorates renal alterations in an animal model of progressive renal tubulointerstitial fibrosis, potentially through inhibiting inflammatory cell infiltration and inducing HGF, resulting in the counteraction of the profibrotic effects of TGF-β1, partly dependent on IP receptor-mediated signaling. The outstanding advantage of SR-ONO treatment over preexisting prostacyclin analogs is its capacity to maintain elevated circulating levels of ONO-1301 accompanied by therapeutic effects, achieved by intermittent administration, potentially leading to improved adherence for patients. We hope that our present study will eventually guide us to the development of novel therapeutic strategies for patients with CKD.

**APPENDIX**

**Preparation of SR-ONO.** ONO-1301 and PLGA (50:50) were dissolved in dichloromethane. The dissolved polymer was added to polyvinyl alcohol aqueous solution to form an oil-in-water emulsion, and then dichloromethane was evaporated by stirring. Following centrifugation and washing, SR-ONO was isolated by lyophilization. The mean particle diameter of SR-ONO was 4.5 ± 0.3 μm as determined by laser diffraction particle size analyzer (model Sald-2100; Shimazu, Kyoto, Japan). The release time was adjusted to 25 days as determined by measuring residual ONO-1301 in the pellets by HPLC (11).

**Immunohistochemistry.** Immunohistochemistry was performed using 4-μm frozen sections as described previously (2, 7, 10, 14). Briefly, frozen sections were fixed in cold (−20°C) acetone for 10 min and exposed to 3% H2O2 to eliminate endogenous peroxidase activity. The kidney sections were then blocked with 10% goat serum for 30 min, and incubated with primary antibody, polyclonal rabbit anti-human FSP-1 (S100A4) antibody (1:100 dilution, A5114; Dakocytomation), and rat anti-mouse F4/80 antibody (1:200 dilution, A3-1; Serotec), for 60 min. The sections were washed with PBS and exposed to secondary antibody, Immun-Star goat anti-rabbit (GAR)-HRP conjugate (1:200 dilution, 170–5046; Bio-Rad), HRP-labeled goat anti-rat IgG (Millipore) for 1 h. Diaminobenzidine was used as a chromogen. All slides were counterstained with hematoxylin. Normal rat IgG was used as a negative control. Renal interstitial monocyte/macrophage infiltration was determined by counting the number of F4/80+ cells in the renal cortex. The number of F4/80+ cells was determined in 15 randomly selected nonoverlapping fields (×400) in each section of the individual mouse renal cortex. The average number of F4/80+ cells from five separate animals was calculated and averaged (13). Similarly, the number of FSP-1+ cells in the tubulointerstitium was determined (×400). For immunohistochemistry of mouse IP receptors, formalin (10%)–fixed, paraffin-embedded sections (4 μm) were used. After deparaffinization, sections were incubated with rabbit polyclonal anti-IP receptor antibody (1:100 dilution; Cayman Chemical) followed by incubation with biotinylated-secondary antibody, and immunoperoxidase staining was carried out utilizing the Vectastain ABC Elite reagent kit (Vector Labs) as previously described (2, 14). Diaminobenzidine was used as a chromogen. All slides were counterstained with hematoxylin. Normal rabbit IgG was used as a negative control.

**Immunofluorescence.** Briefly, frozen sections (4 μm) were fixed in cold (−20°C) acetone for 5–10 min and then air dried. Then, sections were blocked with 10% normal goat serum (426041; Nichirei Biosciences, Tokyo, Japan) followed by incubation with primary antibodies, polyclonal rabbit anti-mouse type I collagen antibody (1:100 dilution, cat. no. AB765-P; Millipore); and polyclonal rabbit anti-mouse type III collagen antibody (1:200 dilution, cat. no. LB-1393; CosmoBio, Tokyo, Japan) for 1 h. Subsequently, the sections were washed three times in PBS and incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG (A11008; Invitrogen, CA) for 1 h. After three washes with PBS, Vectashield antifade mounting medium (Vector Laboratories, Peterborough, UK) was applied, and sections were observed by a fluorescence microscope (model BZ-Analyzer; Keyence), and images were obtained. Normal rabbit IgG were used as negative controls. To assess the type I and type III collagen positive area, the
image files \((1,392 \times 1,040 \text{ pixels})\) at magnification, \( \times 400 \) were analyzed using Lumina Vision software.

**Cell culture.** Briefly, renal cortex was homogenized in radioimmunoprecipitation lysis buffer (cat. no. sc-24948; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C. After centrifugation at 14,000 rpm for 30 min at 4°C, the supernatant was collected and stored at \(-80°C\) until use. The total protein concentration was determined by using the DC-protein determination system (Bio-Rad) using BSA as a standard. Samples were processed for SDS-PAGE, and proteins were electrotransferred onto a nitrocellulose membrane (iBlot Gel transfer stacks, cat. no. IB3010–02; Invitrogen). The membranes were blocked with 5% nonfat dry milk in 1× Tris-buffered saline containing 0.1% Tween-20 for 1 h, incubated overnight with polyclonal rabbit anti-TGF-B1/2/3 (1:500 dilution, cat. no. sc-7892; Santa Cruz Biotechnology), polyclonal rabbit anti-mouse PSmad2/3 (1:1000 dilution, cat. no. sc-11769; Santa Cruz Biotechnology), and rabbit anti-rat HGF (1:1000 dilution; Institute of Immunology, Tokyo, Japan) at 4°C. After incubation with HRP-labeled secondary antibodies (Amersham). The membranes were reprobed with rabbit anti-actin antibodies (1:2,000 dilution, cat. no. A2066; Sigma Aldrich) to serve as controls for equal loading. The density of each band was determined using NIH Image software and expressed as a value relative to the density of the corresponding band obtained from the actin immunoblot.

**Immunofluorescent staining of cultured cells.** mProx24 cells were then fixed with ice-cold acetone for 5 min. The chamber slides were air-dried, immersed in radioimmunoprecipitation lysis buffer at 4°C. After centrifugation at 14,000 rpm for 30 min at 4°C, the supernatant was collected and stored at \(-80°C\) until use. The total protein concentration was determined by using the DC-protein determination system (Bio-Rad) using BSA as a standard. Samples were processed for SDS-PAGE, and proteins were electrotransferred onto a nitrocellulose membrane (iBlot Gel transfer stacks, cat. no. IB3010–02; Invitrogen). The membranes were blocked with 5% nonfat dry milk in 1× Tris-buffered saline containing 0.1% Tween-20 for 1 h, incubated overnight with polyclonal rabbit anti-actin antibodies (1:2000 dilution, cat. no. A2066; Sigma Aldrich) to serve as controls for equal loading. The density of each band was determined using NIH Image software, and expressed as the value relative to the density of the corresponding band obtained from the actin immunoblot.

**RNA extraction and RT-PCR.** Total RNA was extracted from kidneys of each mouse or mProx24 cells using RNeasy Kit (Qiagen, Chatsworth, CA) and stored at \(-80°C\) until use. Total RNA was subjected to RT with poly-d(T) primers and reverse transcriptase (GeneAmp RNA PCR Kit; Applied Biosystems, Foster City, CA). PCR was then carried out using primers for mouse IP receptor and GAPDH (internal control). The program was optimized and performed finally as denaturation at 95°C for 10 min followed by 40 cycles of amplification (mouse IP receptor; 95°C for 15 s, 55°C for 30 s, 72°C for 30 s; GAPDH; 95°C for 10 s, 60°C for 15 s, 72°C for 9 s, respectively). Products were electrophoresed on 1% agarose gel and stained with ethidium bromide. The following oligonucleotide primers specific for mouse IP receptor and GAPDH were used: IP receptor, 5′-TTTCTGCTTCTCTGCCTGACT-3′ (forward) and 5′-ACCCAGACTTGGGGTTGAGAAG-3′ (reverse); GAPDH, 5′-TTGGTCCGTCGTTGACATGA-3′ (forward) and 5′-TTGCTGTT- GAAGTCCGAGAG-3′ (reverse). Four independent experiments were performed.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


