CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

The Selective A₃AR Antagonist LJ-1888 Ameliorates UUO-Induced Tubulointerstitial Fibrosis

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Adenosine in the normal kidney significantly elevates in response to cellular damage. The renal A₃ adenosine receptor (A₃AR) is up-regulated under stress, but the therapeutic effects of A₃AR antagonists on chronic kidney disease are not fully understood. The present study examined the effect of LJ-1888 [(2*R*,3*R*,4*S*)-2-[2-chloro-6-(3-iodobenzylamino)-9*H*-purine-9-yl]-tetrahydrothiophene-3,4-diol], a newly developed potent, selective, species-independent, and orally active A₃AR antagonist, on unilateral ureteral obstruction (UUO)-induced renal fibrosis. Pretreatment with LJ-1888 inhibited UUO-induced fibronectin and collagen I up-regulation in a dose-dependent manner. Masson's trichrome staining confirmed that LJ-1888 treatment effectively reduced UUO-induced interstitial collagen accumulation. Furthermore, delayed administration of LJ-1888 showed an equivalent therapeutic effect on tubulointerstitial fibrosis to that of losartan. Small-interfering A₃AR transfection effectively inhibited transforming growth factor-β1 (TGF-β1)-induced fibronectin and collagen I up-regulation in proximal tubular cells similar to LJ-1888, confirming that the renoprotective effect of LJ-1888 resulted from A₃AR blockade. UUO- or TGF-β1-induced c-Jun N-terminal kinase and extracellular signal-regulated kinase phosphorylation decreased significantly after LJ-1888 administration. A₃AR blockade reduced UUO- or TGF-β1-induced up-regulation of lysyl oxidase, which induces cross-linking of extracellular matrix, suggesting that LJ-1888 may also regulate extracellular matrix accumulation via post-translational regulation. In conclusion, the present data demonstrate that the A₃AR antagonist, LJ-1888, blocked the development and attenuated the progression of renal fibrosis, and they suggest that LJ-1888 may become a new therapeutic modality for renal interstitial fibrosis. (*Am J Pathol* 2013, 183: 1488–1497; <http://dx.doi.org/10.1016/j.ajpath.2013.07.010>)

Chronic kidney disease (CKD) is emerging as a worldwide public health problem. In recent decades, there has been a shift in the major cause of death and disability from nutritional deficiencies and infectious diseases toward progressive chronic diseases. The growing prevalence of CKD is evidence of this shift.¹ However, few effective therapeutic agents, except renin-angiotensin-aldosterone system (RAAS) inhibitors, such as angiotensin II receptor blockers (ARBs)² and angiotensin-converting enzyme inhibitors,³ alone or in combination, are available to treat CKD.^{4,5} Even more serious, these therapies are partially effective at delaying the onset of end-stage renal disease or the decline in glomerular filtration rate, and regression of CKD in humans is rare.⁶ Therefore, newer therapeutic agents to overcome the limitations of RAAS inhibitors are needed.

Adenosine is present in normal kidneys, and its concentration increases dramatically during renal hypoxia and ischemia.⁷ Extracellular adenosine signals through four known adenosine receptor (AR) subtypes, such as A₁, A_{2A}, A_{2B}, and A₃AR. All four ARs are detectable in whole-kidney homogenates, although the exact location of each AR subtype remains to be determined.^{8,9} A recent study¹⁰ reported that increased adenosine in adenosine deaminase (ADA) knockout mice led to

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tubulointerstitial fibrosis, suggesting a pathogenic role for adenosine in CKD. Our previous study showed that all four AR subtypes are up-regulated in the obstructed kidney, which is a well-characterized model of tubulointerstitial fibrosis.¹¹

In this study, we focused on a selective A₃AR antagonist as a novel potent renoprotective agent. Previous studies have reported that mice lacking A₃AR or wild-type mice, in which the A₃AR is blocked pharmacologically, show significant protection against acute renal injury, such as ischemia-reperfusion injury and myoglobinuria-induced injury.^{12,13} However, the role of A₃AR in CKD and the mechanisms involved are not fully understood. Because all four AR subtypes are members of G-protein-coupled receptors (the Gi-coupled A₁AR and A₃AR versus the Gs-coupled A_{2A}AR and A_{2B}AR), AR signaling occurs through inhibition or stimulation of adenylyl cyclase. However, other pathways, such as phospholipase C, Ca²⁺, and mitogen-activated protein kinases (MAPKs), are also relevant.¹⁴ MAPK is one among the various intracellular signaling pathways involved with A₃AR.^{13,15} It is well established that MAPKs, including c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 MAPK, are involved in tubulointerstitial fibrosis,^{16–18} which is characterized by increased extracellular matrix (ECM) accumulation and the epithelial-to-mesenchymal transition (EMT) and is a common feature of all kidney diseases leading to chronic renal failure.¹⁹ Because of the relationship between the EMT and MAPK, A₃AR may play a role in EMT and ECM accumulation through MAPK.²⁰

This study was performed to investigate the therapeutic effect of LJ-1888 [(2*R*,3*R*,4*S*)-2-[2-chloro-6-(3-iodobenzylamino)-9*H*-purine-9-yl]-tetrahydrothiophene-3,4-diol], a newly developed potent, selective, species-independent, and orally active A₃AR antagonist,²¹ on unilateral ureteral obstruction (UUO)-induced tubulointerstitial fibrosis. We further investigated whether the anti-fibrotic effect of LJ-1888 occurs via A₃AR deficiency using A₃AR-specific siRNA (siA₃AR)-transfected murine proximal tubular (mProx) cells cultured with transforming growth factor-β1 (TGF-β1), and evaluated the possible renoprotective mechanism of LJ-1888.

Materials and Methods

Animals and the UUO Model

All animal experiments were approved by the Institutional Animal Care and Use Committee (No. 2010-5-6) at Ewha Womans University (Seoul, Korea). The UUO model was established in 8-week-old male Sprague-Dawley rats (Central Lab Animal Inc., Seoul, Korea). UUO was conducted under 10 mg/kg tiletamine/zolazepam (Virbac Laboratories, Carros, France)-induced anesthesia. Briefly, anesthetized rats were placed on a heating pad at 37°C. The left ureter was visualized after a flank incision, ligated with silk (4/0) at two locations, and cut between ligatures to prevent a urinary tract infection. The right ureter was subjected to the same surgical procedures, but the ureter was not ligated.

Series I, Preventive Effect of LJ-1888

To examine the preventive effect of LJ-1888 on the progression of renal fibrosis, LJ-1888 was administered daily by oral gavage at doses of either 1 or 10 mg/kg. The control group was administered an equal volume of 0.25% carboxymethyl cellulose by oral gavage. Medication was started 5 days before surgery in all groups to allow for a sufficient drug effect and was continued until sacrifice. The rats were sacrificed at 5 days after UUO induction.

Series II, Therapeutic Effect of LJ-1888

LJ-1888 was administered on day 3 after obstruction to examine the effect of LJ-1888 on progressive renal fibrosis. The preliminary time-course study of UUO showed that α-smooth muscle actin (α-SMA) and fibronectin (FN) mRNA expressions and collagen accumulation in the obstructed kidneys were statistically increased at 3 days after ligation ([Supplemental Figure S1, A–D](#)). Groups 1 and 2 were sham operated on and UUO, respectively, and rats were sacrificed at 3 days after surgery. Groups 3 and 4 included rats that underwent UUO injury and were given either vehicle or 10 mg/kg LJ-1888, respectively, from days 3 to 10 (7 days). In addition, groups 5 and 6 were rats that underwent UUO injury and were given either 10 mg/kg losartan or combination therapy with 10 mg/kg each of LJ-1888 and losartan, respectively, from days 3 to 10 (7 days) to compare the effect between LJ-1888 and losartan. All medication was given daily by oral gavage and continued until sacrifice.

Cell Culture

Immortalized mProx cells, derived from microdissected proximal tubular segments of C57BL/6J adult murine kidneys, were supplied by Dr. Takeshi Sugaya (St Marianna University School of Medicine, Kanagawa, Japan). mProx cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA), 100 U/mL penicillin, 100 µg/mL streptomycin, and 44 mmol/L NaHCO₃, under a 5% CO₂ environment at 37°C. The cultured cells grown to confluence were growth arrested with Dulbecco's modified Eagle's medium containing 0.15% fetal bovine serum for 24 hours before commencing experiments. Subconfluent mProx cells were transfected with 50 nmol/L siA₃AR using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) for 24 hours.

Real-Time and RT-PCR

Total RNA was extracted from tissues or cells using TRIzol (Invitrogen), and real-time quantitative RT-PCR with 18s rRNA as an internal control was performed using the SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA) and an ABI 7300 Real-Time PCR Thermal Cycler (Applied Biosystems). The quantities of the test gene and

internal control 18s rRNA were obtained from standard curves using Applied Biosystems software version 1.4.0. mRNA expression levels of test genes were normalized to 18s rRNA levels. The primer sequences for each gene are listed in Table 1.

Western Blot Analysis

The relative protein expression was analyzed using Western blot analysis, as previously described.²² Briefly, kidney tissues or cells were lysed in Laemmle lysis buffer, mixed with a 5× sample buffer [60 mmol/L Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 0.1% bromophenol, and 0.2% 2-mercaptoethanol], and heated at 95°C for 10 minutes. The lysates were separated using SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed using various antibodies, including anti- α -SMA (Sigma-Aldrich, St. Louis, MO), anti-E-cadherin (BD Bioscience, San Jose, CA), anti-FN (Santa Cruz Biotechnology, Santa Cruz, CA), anti-collagen I (Col I; Southern Biotech, Birmingham, AL), anti-phospho-JNK (Cell Signaling Technology, Danvers, MA), anti-total JNK (Cell Signaling Technology), anti-phospho-ERK (Cell Signaling Technology), anti-total ERK (Cell Signaling Technology), anti-phospho-Smad3 (Cell Signaling Technology), and anti-total Smad3 (Cell Signaling Technology). The blots were reacted with secondary antibodies, followed by enhanced chemiluminescence, and then imaged using LAS-3000 film (Fuji, Tokyo, Japan). Positive immunoreactive bands were quantified by densitometry, normalized to β -actin, and compared with those of the control.

Histological Analysis

Kidneys were removed from sacrificed rats, sliced vertically, and postfixed with 2% paraformaldehyde-lysine-periodate, pH 7.4. After routine processing through a graded alcohol series and 100% xylene, the kidneys were embedded in paraffin and sections (4 μ m thick) were divided. Sections were stained with Masson's modified trichrome to demonstrate collagen matrix accumulation. Each slide was stained using a Masson's trichrome staining kit (HT15-1KT), according to the manufacturer's protocol. The Masson's trichrome staining positive area was quantitatively measured using ImageJ software version 1.47 (NIH, Bethesda, MD).

Data Analysis

All results are expressed as means $\pm$ SEM, with N as the number of experiments. Each group was compared by analysis of variance and Tukey's post hoc analysis. $P < 0.05$ was considered significant.

Results

LJ-1888 Prevents Tubulointerstitial Fibrosis in Obstructed Kidneys

EMT and ECM accumulation is a key feature of tubulointerstitial fibrosis. α -SMA and E-cadherin expression levels were measured as EMT markers. α -SMA mRNA and protein expression levels increased after UUO and were

Table 1 Primer Sequences

Primers	Forward	Reverse
Rat		
18s rRNA	5'-AGGAATTGACGGAAGGGCAC-3'	5'-GTGCAGCCCCCGACATCTAAG-3'
A ₁ AR	5'-AGTACTTCTGGGGTCAACCG-3'	5'-CCATGTTTGGCTGGAACAAC-3'
A ₃ AR	5'-CTTCAGAGAGACGCGTGCAT-3'	5'-TGCAGGCGTAGACAATAGGG-3'
A _{2A} AR	5'-CGGGATCCGTCCCTGGCCATCATCGT-3'	5'-GGAATTCCCCCTCAGTGCTGTGAGC-3'
A _{2B} AR	5'-AGTAAAGACCGTGCCACCAG-3'	5'-GGAAGGACACACCCAAAGAA-3'
α -SMA	5'-GATCACCATCGGGAATGAACGC-3'	5'-CTTAGAAGCATTTGCGGTGGA-3'
Collagen I	5'-TCTAAGACATCCCTGGTCAC-3'	5'-GTCCTTCCAGAAGAAACCTT-3'
E-cadherin	5'-CTGATTCTGATCCTCTGCTCCT-3'	5'-ATCATTTTGAATCACTTCCGGT-3'
Fibronectin	5'-TTATGACGACGGGAAGACCT-3'	5'-GCTGGATGGAAGATTACTC-3'
Lysyl oxidase	5'-TCCACGTACGTACAAAAGATGTCTA-3'	5'-GTAGCAGTACCTGTGGTCATAGTC-3'
TGF- β 1	5'-CCAACTACTGCTTCAGCTCCA-3'	5'-GTGTCCAGGCTCCAAATGT-3'
Mouse		
18s rRNA	5'-AAACGGCTACCACATCCAAG-3'	5'-CCTCCAATGGATCCTCGTTA-3'
A ₁ AR	5'-GTGATTTGGGCTGTGAAGGT-3'	5'-AGTAGGTCTGTGGCCCAATG-3'
A _{2A} AR	5'-GGCTCCTCGGTGTACATCAT-3'	5'-GTTCTGCAGGTGTGCTGTTGA-3'
A _{2B} AR	5'-GCGAATAAAAGCTGCTGTCTC-3'	5'-AGGCCACCATGAAGATTTTG-3'
A ₃ AR i1	5'-GGACTGGCTGAACATCACT-3'	5'-AGCCAGTGCTAGGGAGACAA-3'
A ₃ AR i2	5'-GGCCAGAGATGACATGGATT-3'	5'-CAGCTTTGGAAGCCCTACAG-3'
A ₃ AR i1/3	5'-CCCTGGTTGTCTGTGTGTC-3'	5'-TGTCTTGAACCTCCGTCAT-3'
α -SMA	5'-GTCCAGACATCAGGGAGTAA-3'	5'-TCGGATACTTCAGCGTCAGGA-3'
Collagen I	5'-CGGATAGCAGATTGAGAACATCCG-3'	5'-CGGCTGAGTACGGAACACACA-3'
Fibronectin	5'-CGGCGTATGCTGTCACTGGCCG-3'	5'-AAGTTGAAGGCACCCACCTG-3'
Lysyl oxidase	5'-CACACACAGGGATTGATCCT-3'	5'-AGCTGGGGTTTACTACTGACCT-3'

Figure 1 Pretreatment effect of LJ-1888 on epithelial-to-mesenchymal transition (EMT) in obstructed kidneys. α -SMA (A and B) and E-cadherin (C and D) expression levels were measured to evaluate the effect of LJ-1888 on EMT in obstructed kidneys. mRNA expression was measured by real-time PCR (A and C), and protein expression was measured by using Western blot analysis (B and D). Data are means $\pm$ SEM of six rats per group. * P < 0.05 versus sham.

inhibited by 10 mg/kg per day LJ-1888 (Figure 1, A and B). E-cadherin mRNA tended to increase in LJ-1888-treated obstructed kidneys (Figure 1C), but E-cadherin protein expression remained to be unchanged (Figure 1D). UUO-induced mRNA and protein expression levels of Col I and FN (Figure 2, A–D) decreased significantly after LJ-1888 treatment. In particular, Col I protein expression decreased to the basal level (Figure 2B). Masson's trichrome staining was also used to evaluate the pretreatment effect of LJ-1888 on ECM accumulation (Figure 2, E and F). Excessive interstitial collagen deposition, tubular atrophy, and dilation were observed in the vehicle-treated obstructed kidneys, and LJ-1888 treatment effectively reduced collagen accumulation in a dose-dependent manner.

Delayed Administration of LJ-1888 Inhibits Tubulointerstitial Fibrosis in Obstructed Kidneys

Because patients with CKD are usually diagnosed after renal fibrosis initiation, it would be useful if LJ-1888 reverses or slows the progression of renal fibrosis after tubulointerstitial fibrosis initiation. Therefore, the administration of LJ-1888 was initiated at 3 days after UUO induction and continued until 10 days after UUO induction to evaluate the therapeutic effect of delayed administration of LJ-1888 on the progression of tubulointerstitial fibrosis caused by UUO injury.²³ We also compared the effect of LJ-1888 with that of losartan, a prototypic renoprotective agent.

Figure 2 Pretreatment effect of LJ-1888 on ECM accumulation in obstructed kidneys. Col I (A and B) and FN (C and D) expression levels were measured to evaluate the effect of LJ-1888 on ECM accumulation in obstructed kidneys. mRNA expression was measured by real-time PCR (A and C), and protein expression was measured by using Western blot analysis (B and D). Masson's trichrome staining was performed (E), and positive staining area was quantified by using ImageJ software version 1.47 from 10 random cortical fields per rat to evaluate the pretreatment effect of LJ-1888 on collagen accumulation in obstructed kidney (F). Original magnification, $\times 100$ (E). Data are means $\pm$ SEM of six rats per group. * P < 0.05 versus sham; $^{\dagger}P$ < 0.05 versus vehicle-treated UUO; and $^{\ddagger}P$ < 0.05 versus 1 mg/kg LJ-1888-treated UUO.

Figure 3 Effect of delayed LJ-1888 administration on EMT in progressive obstructed kidneys. α -SMA (A and B) and E-cadherin (C and D) expression levels were measured to evaluate the therapeutic effect of LJ-1888 on the EMT in progressively obstructed kidneys. mRNA expression was measured by real-time PCR (A and C), and protein expression was measured by using Western blot analysis (B and D). LJ, 10 mg/kg LJ-1888 treated; LJ + LO, both 10 mg/kg LJ-1888 and 10 mg/kg losartan treated; LO, 10 mg/kg losartan treated; V, vehicle treated; S, sham; U, unilateral ureteral obstruction. Data are means $\pm$ SEM of six rats per group. * P < 0.05 versus sham.

UUO-induced elevation in α -SMA mRNA and protein expression was not reduced by 10 mg/kg of either LJ-1888 or losartan (Figure 3, A and B). E-cadherin mRNA expression did not decrease in the obstructed kidneys (Figure 3C), but E-cadherin protein expression decreased significantly at 10 days after UUO, which tended to be reversed by combination therapy with LJ-1888 and losartan, although it did not reach statistical significance (Figure 3D).

Col I and FN expression levels were measured to evaluate the effect of delayed administration of LJ-1888 on the progression of tubulointerstitial fibrosis. Col I and FN mRNA expression significantly increased in obstructive kidneys from day 3 after UUO induction compared with those in sham kidneys, which were not significantly affected by LJ-1888, losartan, or combination therapy (Figure 4, A and C). However, UUO-induced Col I and FN protein expression

decreased by delayed treatment with LJ-1888 or losartan alone and combination with both agents (Figure 4, B and D). Masson's trichrome staining also revealed that delayed treatment with LJ-1888, losartan, and combination therapy effectively reduced the collagen accumulation in the interstitial area induced by UUO (Figure 4, E and F). These data suggest that delayed administration of LJ-1888 is effective for blocking the progression of UUO-induced tubulointerstitial fibrosis if the treatment starts within a certain time during the disease process.

TGF- β 1-Induced Col I and FN mRNA Expression Levels Decrease in A₃AR-Suppressed mProx Cells

A₃AR-specific siRNA and structural analogues of LJ-1888 were used in TGF- β 1-treated mProx cells to confirm that

Figure 4 Effect of delayed LJ-1888 administration on ECM accumulation in progressive obstructed kidneys. Col I (A and B) and FN (C and D) expression levels were measured to evaluate the therapeutic effect of LJ-1888 on ECM accumulation in progressive obstructed kidneys. mRNA expression was measured by real-time PCR (A and C), and protein expression was measured by using Western blot analysis (B and D). Masson's trichrome staining was performed (E), and positive staining area was quantified by using ImageJ software version 1.47 from 10 random cortical fields per rat to evaluate the therapeutic effect of LJ-1888 on collagen accumulation in obstructed kidneys (F). LJ, 10 mg/kg LJ-1888 treated; LJ + LO, both 10 mg/kg LJ-1888 and 10 mg/kg losartan treated; LO, 10 mg/kg losartan treated; V, vehicle treated. Data are means $\pm$ SEM of six rats per groups. * P < 0.05 versus sham, † P < 0.05 versus vehicle treated.

LJ-1888 has an anti-fibrotic renoprotective effect in an A₃AR-dependent manner. TGF- β 1 significantly induced Col I and FN mRNA up-regulation. LJ-1888 effectively inhibited up-regulation of TGF- β 1-induced Col I and FN mRNA (Figure 5, A and B). Knocking down A₃AR expression in mProx cells to 50% with A₃AR-specific siRNA (Figure 5C) effectively inhibited TGF- β 1-induced Col I and FN mRNA expression (Figure 5, D and E). LJ-1888 and other LJ compound series A₃AR antagonists (Supplemental Table S1) also inhibited TGF- β 1-induced Col I synthesis in mProx cells (Supplemental Figure S2). However, LJ-2525 and LJ-2526, which are 4'-oxo analogues of LJ-1888, did not reduce TGF- β 1-induced Col I mRNA expression because they showed much lower binding affinity than that of LJ-1888 and did not inhibit A₃AR activation. These results indicate that the anti-fibrotic effects of LJ-1888 occur via an A₃AR-dependent pathway.

JNK and ERK Phosphorylation Are Inhibited by Inhibiting A₃AR

Phosphorylation of MAPK, which is related to EMT and ECM accumulation,²⁴ was analyzed to examine the mechanism involved in renoprotective effect of LJ-1888 on tubulointerstitial fibrosis. Phosphorylation of JNK and ERK, but not p38 MAPK (data not shown), increased in obstructed kidneys compared with that in sham kidneys. UUO-induced JNK (Figure 6A) and ERK (Figure 6B) phosphorylation was inhibited by 10 mg/kg LJ-1888. TGF- β 1 also induced JNK and ERK phosphorylation in mProx cells, which was effectively inhibited by 20 μ mol/L LJ-1888 (Figure 6, C and D).

LJ-1888 Does Not Affect UUO-Induced TGF- β 1 Up-Regulation and TGF- β 1-Induced Smad Signaling in mProx Cells

We next measured TGF- β 1 mRNA expression and Smad3 phosphorylation, because the TGF- β 1/Smad axis is the major signaling pathway in progression of the EMT and ECM accumulation. TGF- β 1 mRNA expression increased in obstructed kidneys, and neither pretreatment nor delayed treatment with LJ-1888 affected UUO-induced TGF- β 1 up-regulation (Figure 7, A and B). Smad3 phosphorylation increased significantly, suggesting functional up-regulation of TGF- β 1 in obstructed kidneys, but total Smad3 also increased in obstructed kidneys. LJ-1888 did not alter either Smad3 phosphorylation or total Smad3 in the obstructed kidney (Figure 7C). LJ-1888 pretreatment significantly, but partially, inhibited TGF- β 1-induced Smad3 phosphorylation in mProx cells (Figure 7D).

Suppression of A₃AR May Reduce ECM Accumulation via Regulation of Lysyl Oxidase Expression

Because LJ-1888 did not show a significant effect on EMT progression in obstructed kidneys, despite the effective

reduction in Col I and FN, we measured the level of lysyl oxidase (LOx), which is important for cross-linking of ECM protein.²⁵ Pretreatment with LJ-1888 effectively reduced UUO-induced LOx mRNA up-regulation (Figure 8A), although delayed administration of LJ-1888, losartan, or a combination of the two agents did not affect UUO-induced LOx up-regulation (Figure 8B). Suppressing A₃AR via LJ-1888 (Figure 8C) or siA₃AR (Figure 8D) also significantly inhibited TGF- β 1-induced LOx up-regulation in mProx cells.

Discussion

We presented experimental data demonstrating that a species-independent and orally active A₃AR antagonist blocks the development of tubulointerstitial fibrosis when given before UUO induction and attenuates the progression of renal fibrosis when administered after tubulointerstitial fibrosis has initiated.

UUO is a well-defined animal model for tubulointerstitial fibrosis. Tubular epithelial cells differentiate into myofibroblasts in the obstructed kidney and express α -SMA and ECM proteins (FN and Col I).²⁶ Consistent with previous reports, α -SMA and ECM proteins were effectively induced in obstructed kidneys. Loss of epithelial adhesion is a major key event during the EMT, but E-cadherin expression did not decrease at day 5 of UUO (Figure 1, C and D) but did occur at day 10 of UUO (Figure 3D) in the present study. The reason why E-cadherin mRNA and protein expression at day 5 of UUO did not decrease is unclear. A previous study reported

Figure 5 Effect of A₃AR blockade on TGF- β 1-induced profibrotic markers in mProx cells. The effects of LJ-1888 on the TGF- β 1-induced Col I (A) and FN (B) mRNA expression in mProx cells, a mouse proximal tubular epithelial cell line, were measured by real-time PCR. The efficacy of the siA₃AR was measured by real-time PCR (C). mProx cells were transfected with 50 nmol/L siA₃AR or scrambled siRNA (siScr) for 24 hours. Transfected mProx cells were stimulated with TGF- β 1 for 6 hours. The effect of siA₃AR on TGF- β 1-induced Col I (D) and FN (E) mRNA expression was measured. Data are means $\pm$ SEM of four experiments. * P < 0.05 versus control; $^{\dagger}P$ < 0.05 versus 10 ng/mL TGF- β 1 (A and B). * P < 0.05 versus siScr-transfected cells, $^{\dagger}P$ < 0.05 versus siScr-transfected cells treated with TGF- β 1 (C–E).

Figure 6 Effect of LJ-1888 on UUO- or TGF- β 1-induced JNK and ERK phosphorylation. The effect of LJ-1888 on JNK (A) and ERK (B) phosphorylation in obstructed kidneys was measured by using Western blot analysis, quantified by densitometry, and normalized to total JNK or ERK. Data are means $\pm$ SEM of six rats per group. * P < 0.05 versus sham, $^{\dagger}P$ < 0.05 versus vehicle-treated UUO (A and B). mProx cells were pretreated with or without 20 μ M LJ-1888 for 30 minutes before adding 10 ng/mL TGF- β 1. At 15 minutes after the treatment with TGF- β 1, JNK (C) and ERK (D) phosphorylation was measured. Data are means $\pm$ SEM of four experiments. * P < 0.05 versus control; $^{\dagger}P$ < 0.05 versus 10 ng/mL TGF- β 1 (C and D).

that E-cadherin mRNA and protein expression increased at 3 and 10 days after UUO induction in Wistar rats weighing 200 to 250 g (2 months).²⁷ In addition, the loss of E-cadherin protein expression was detected after 7 days of UUO in CD-1 mice, but E-cadherin tended to increase in obstructed kidneys at an early stage, such as 1 or 3 days after UUO induction.²⁸ Taken together, these results suggest that loss of E-cadherin may depend on the timing relative to obstruction and on the species under investigation.

We showed that all four AR subtypes were up-regulated in obstructed kidneys, which was consistent with studies reporting that various cellular distress conditions, such as ischemia, hypoxia, or inflammation, significantly alter AR expression.^{11,29} The rat genome has only one Adora3 isoform, but the mouse genome has three Adora3 isoforms encoding A₃AR. Adora3i1 and Adora3i2 share no coding regions, whereas Adora3i3 shares coding exons only with Adora3i1.³⁰ mRNA expression of ARs in the present study

(Supplemental Figure S3) was correlated with the time course of UUO injury, and TGF- β 1 significantly increased A₃AR i1 and 1/3 mRNA expression in mProx cells (Supplemental Figure S4), suggesting that increased A₃AR expression is related to obstructed renal injury, including tubulointerstitial fibrosis. Although the renoprotective effects of A₃AR antagonists have been reported, previous studies focused on acute kidney injury.^{12,13} The role of adenosine signaling in renal fibrosis during CKD has been suggested, based on the observation that increasing adenosine as a result of ADA deficiency significantly induces renal fibrosis in ADA knockout mice.¹⁰ However, that study emphasized A_{2B}AR as a mediator of renal fibrosis. To our knowledge, the present study is the first that has focused on A₃AR as a CKD target.

Pretreatment of 10 mg/kg LJ-1888 reduced A₃AR up-regulation in obstructed kidneys (Supplemental Figure S5A) but delayed administration of either LJ-1888 or losartan alone or in combination did not affect A₃AR mRNA up-regulation (Supplemental Figure S5B). These results indicate that A₃AR blockade can prevent, but not reverse, UUO-induced A₃AR up-regulation. On the other hand, obstructed kidney exhibits a hypoperfusion state and proper concentration of drugs may not be reached in the injured kidney.

Underlying mechanisms of obstructed renal injury are multifactorial, and a mechanical stretch from urine

Figure 7 Effect of LJ-1888 on UUO-induced TGF- β 1 expression or TGF- β 1-induced Smad3 phosphorylation. TGF- β 1 mRNA expression in preventive (A) or therapeutic (B) effect of LJ-1888 was measured by real-time PCR. Data are means $\pm$ SEM of six rats per group. * P < 0.05 versus sham (A and B). C: The effect of LJ-1888 on Smad3 phosphorylation in obstructed kidneys was measured by using Western blot analysis. Data are means $\pm$ SEM of six rats per group. * P < 0.05 versus sham (C). mProx cells, a mouse proximal tubular epithelial cell line, were pretreated with or without 20 μ M LJ-1888 for 30 minutes before adding 10 ng/mL of TGF- β 1. D: At 30 minutes after the treatment with TGF- β 1, the effect of LJ-1888 on Smad3 phosphorylation was measured. Data are means $\pm$ SEM of four experiments. * P < 0.05 versus control; $^{\dagger}P$ < 0.05 versus 10 ng/mL TGF- β 1 (D).

Figure 8 Effect of A₃AR blockade on UUO- or TGF-β1-induced LOx up-regulation. LOx mRNA expression in preventive (A) or therapeutic (B) effect of LJ-1888 was measured by real-time PCR. Data are means ± SEM of six rats per group. *P < 0.05 versus sham, †*P* < 0.05 versus vehicle-treated UUO group (A and B). C: The effect of LJ-1888 on the TGF-β1-induced LOx mRNA expression in mProx cells, a mouse proximal tubular epithelial cell line, was measured. Data are means ± SEM of four experiments. *P < 0.05 versus control, †*P* < 0.05 versus 10 ng/mL TGF-β1 (C). mProx cells were transfected with 50 nmol/L siA₃AR or scrambled siRNA (siScr) for 24 hours. D: Transfected mProx cells were stimulated with TGF-β1 for 6 hours. The effect of siA₃AR on TGF-β1-induced LOx mRNA expression was measured. Data are means ± SEM of four experiments. *P < 0.05 versus siScr-transfected cells, †*P* < 0.05 versus siScr-transfected cells treated with TGF-β1 (D). LJ, 10 mg/kg LJ-1888 treated; LJ + LO, both 10 mg/kg LJ-1888 and 10 mg/kg losartan treated; LO, 10 mg/kg losartan treated; V, vehicle treated.

accumulation, angiotensin II, TGF-β1, and inflammatory cytokines has been proposed.²⁶ It is, therefore, important to target simultaneously more than one pathological receptor/pathway for effective therapy aiming to stop progression of renal fibrosis in patients with CKD. Most of these factors mainly act via G-protein-coupled receptors or tyrosine kinase receptors whose downstream signaling involves various effectors, including MAPK. The MAPK family uses three parallel signaling pathways, such as JNK, ERK, and p38 MAPK. It is well established that phosphorylations of JNK, ERK, and p38 MAPK all increase and are involved in the EMT and ECM accumulation in obstructed kidneys.^{31–33} Consistent with previous studies, JNK and ERK phosphorylation increased, whereas p38 MAPK phosphorylation did not increase in vehicle-treated obstructed kidneys in the present study. The reason why p38 MAPK phosphorylation did not increase in obstructed kidneys under the present conditions is unclear. LJ-1888 significantly reduced JNK and ERK phosphorylation in obstructed kidneys (Figure 6, A and B) and in mProx cells cultured with TGF-β1 (Figure 6, C and D). Cross talk between MAPK and Smad, a canonical TGF-β1 signaling pathway, has been reported in tissue

fibrosis,^{16,34,35} but LJ-1888 did not show any effect on UUO-induced Smad3 phosphorylation (Figure 7C) and TGF-β1 mRNA up-regulation (Figure 7, A and B) in obstructed kidneys. LJ-1888 partially inhibited TGF-β1-induced Smad3 phosphorylation in mProx cells (Figure 7D). Therefore, inhibiting JNK and ERK may account, at least in part, for the anti-fibrotic effect of LJ-1888, and LJ-1888 may have an anti-fibrotic effect at the presence of TGF-β1 up-regulation in the kidneys.

Another mechanism involved in renal fibrosis is the role of infiltrated macrophages through secreting many cytokines contributing to fibroblast activation and myofibroblast generation.^{19,26,36} Monocyte chemoattractant protein-1 (MCP-1) mRNA expression was increased in obstructed kidneys, but either pretreatment (Supplemental Figure S6A) or delayed administration (Supplemental Figure S6B) of LJ-1888 did not alter UUO-induced MCP-1 up-regulation. Consistent with MCP-1 data, we did not find a significant effect of LJ-1888 on increased ectodysplasin A (ED-1)-positive staining in obstructed kidneys (Supplemental Figure S6, C and D). Because the anti-inflammatory effect of A₃AR agonists has been suggested,^{7,14} the exact effect/mechanism of A₃AR on renal inflammation remains to be studied. Yet, it is important to appreciate that LJ-1888 may have an antifibrotic effect at the presence of macrophage infiltration in obstructed kidneys. To further evaluate the antifibrotic mechanism of LJ-1888 on UUO-induced ECM accumulation, LOx expression was measured. LOx plays a major role in the formation and stabilization of the ECM by oxidizing lysine residues and initiating the formation of covalent cross-linkages.²⁵ Consistent with a previous study,³⁷ LOx is increased in obstructed kidneys (Figure 8). LJ-1888 effectively prevented UUO-induced LOx mRNA expression (Figure 8A), and A₃AR blockade, by either LJ-1888 or siA₃AR, significantly inhibited TGF-β1-induced LOx up-regulation in the mProx cells (Figure 8, C and D), suggesting that the renoprotective effect of LJ-1888 may contribute to the inhibition of cross-linking of ECM through reduction of LOx.

Delayed administration of LJ-1888 also effectively reduced tubulointerstitial fibrosis in the obstructed kidneys (Figures 3 and 4). RAAS inhibitors, such as angiotensin-converting enzyme inhibitors and ARBs, are the first-line drugs for treating renal fibrosis.⁴ In this study, the therapeutic effect of LJ-1888 was compared with that of losartan, an ARB, as a positive control. LJ-1888 showed similar therapeutic effects against renal fibrosis as losartan. UUO-induced Col I and FN protein expression decreased significantly after LJ-1888 or losartan treatment, alone or in combination. Previous preventive studies reported that RAAS inhibitors reduce ECM protein's mRNA expression,^{38,39} which was not shown in the present intervention study. It is unclear why LJ-1888 or losartan alone did not affect Col I and FN mRNA expression, but there are some possibilities. First, the dosage we used may have been insufficient to regulate mRNA transcription in hypoperfusion status of obstructed kidneys, as previously discussed. More than 20 mg/kg ARB is usually used to

evaluate their preventive effect on renal fibrosis.^{40–42} Second, effective inhibition of ECM protein without any effect on mRNA expression could be explained through increased degradation of ECM proteins.⁴³ Gelatin gel zymography showed that matrix metalloproteinase-9 (MMP-9) activity was decreased in 10 days after the obstruction, which was effectively inhibited by LJ-1888 or losartan alone or in combination (Supplemental Figure S7). Further studies are, however, needed to determine the precise role of A₃AR in MMP activity and ECM degradation in the obstructed kidneys, because the role of MMP in the obstructed kidneys has been controversial as the result of complexity of proteolytic systems in tubulointerstitial fibrosis of the obstructed kidneys. The MMP-9 activity of the obstructed kidneys was either decreased⁴⁴ or increased.⁴⁵

The effects of the A₃AR antagonist, LJ-1888, and other LJ compounds on renal fibrosis were confirmed using cell culture. A₃AR antagonists effectively reduced TGF-β1–induced Col I mRNA expression (Supplemental Figure S2). Furthermore, siA₃AR transfection targeting the A₃AR isoform 1/3, which is up-regulated under TGF-β1 conditions, significantly inhibited TGF-β1–induced Col I and FN mRNA up-regulation (Figure 5). These results show that the therapeutic effect of A₃AR antagonists occurs by blocking A₃AR activity.

In summary, the present data demonstrate that the A₃AR antagonist, LJ-1888, blocked the development of tubulointerstitial fibrosis and attenuated the progression of renal interstitial fibrosis, and they suggest that LJ-1888 may become a new therapeutic modality for renal interstitial fibrosis.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.07.010>.

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