

LPA₁ Receptor Activation Promotes Renal Interstitial Fibrosis

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ABSTRACT

Tubulointerstitial fibrosis in chronic renal disease is strongly associated with progressive loss of renal function. We studied the potential involvement of lysophosphatidic acid (LPA), a growth factor–like phospholipid, and its receptors LPA_{1–4} in the development of tubulointerstitial fibrosis (TIF). Renal fibrosis was induced in mice by unilateral ureteral obstruction (UUO) for up to 8 d, and kidney explants were prepared from the distal poles to measure LPA release into conditioned media. After obstruction, the extracellular release of LPA increased approximately 3-fold. Real-time reverse transcription PCR (RT-PCR) analysis demonstrated significant upregulation in the expression of the LPA₁ receptor subtype, downregulation of LPA₃, and no change of LPA₂ or LPA₄. TIF was significantly attenuated in LPA₁ (–/–) mice compared to wild-type littermates, as measured by expression of collagen III, α -smooth muscle actin (α -SMA), and F4/80. Furthermore, treatment of wild-type mice with the LPA₁ antagonist Ki16425 similarly reduced fibrosis and significantly attenuated renal expression of the profibrotic cytokines connective tissue growth factor (CTGF) and transforming growth factor β (TGF β). *In vitro*, LPA induced a rapid, dose-dependent increase in CTGF expression that was inhibited by Ki16425. In conclusion, LPA, likely acting through LPA₁, is involved in obstruction-induced TIF. Therefore, the LPA₁ receptor might be a pharmaceutical target to treat renal fibrosis.

J Am Soc Nephrol 18: 3110–3118, 2007. doi: 10.1681/ASN.2007020196

The incidence of chronic kidney disease leading to end-stage renal disease (ESRD) continues to increase throughout the world.¹ Almost all forms of ESRD are preceded by the progressive appearance of renal fibrosis (*i.e.*, extracellular matrix (ECM) accumulation). The presence of fibrosis in the tubulointerstitium (*i.e.*, TIF), compared with glomerular sclerosis, correlates strongly with evolution toward ESRD.^{1,2} The development of TIF can be schematically divided: (1) Inflammation associated with infiltration of macrophages, lymphocytes, and an increase in circulating cytokines and chemokines. (2) This inflammation induces disequilibrium between apoptosis and proliferation of tubular cells, as well as accumulation of myofibroblasts.

Myofibroblasts infiltrate from the circulation into the interstitium, appear by epithelial mesenchymal transition (EMT), or appear by proliferation/activation of the few resident fibroblasts. (3) These myofibroblasts are the main cell type responsible

Received February 13, 2007. Accepted July 6, 2007.

Published online ahead of print. Publication date available at www.jasn.org.

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for the secretion of the ECM.^{1,3} As these events occur, the amount of fibrotic tissue increases, causing a steady decline of renal function until eventually the kidney is no longer able to function and organ failure occurs.

In the past, a number of mediators of TIF development have been identified, including chemokines, cytokines, and growth factors.⁴ Among these, TGF β is thought to be the most fibrogenic, directly or indirectly through the action of CTGF.⁵

LPA is a growth factor–like phospholipid known to regulate several cellular processes including motility, proliferation, survival, and differentiation by acting *via* specific G-protein–coupled receptors (LPA₁, LPA₂, LPA₃, and LPA₄).⁶ Until now, a limited number of pharmacological tools specifically targeting LPA receptor subtypes have been developed. Among them is the antagonist Ki16425, which has been demonstrated to specifically block LPA₁ and LPA₃ receptor subtypes *in vitro*.⁷ Recently, the *in vivo* efficacy of Ki16425 in blocking the action of the LPA₁ receptor subtype has been demonstrated.⁸ LPA has been associated with the etiology of a growing number of disorders,⁹ but the involvement of LPA in the progression to ESRD is unclear. In acute renal disease, contradictory results were obtained since intraperitoneal injection of LPA was reported to prevent renal ischemia-reperfusion injury,¹⁰ whereas pharmacologic blockade of LPA₃ receptor was reported to reduce renal ischemia-reperfusion injury.¹¹ However, in patients with chronic renal failure, it has been reported that LPA concentrations are increased.^{12,13} These observations led us to hypothesize that LPA could be involved in the response of the kidney to injuries and could thus contribute to the progression of chronic renal disease.

The objective of our study was to clearly determine the contribution of LPA in the development of TIF, a hallmark of progressive renal disease. We studied LPA production and the expression of LPA receptor subtypes in kidneys subjected to UUO, an accelerated model of TIF.^{3,14} We observed that UUO-induced renal TIF is accompanied by an upregulation of LPA₁ receptor expression and by an increased release of LPA by the obstructed kidney, UUO-induced fibrosis is significantly attenuated in kidneys from LPA₁($-/-$) mice as well as in mice treated with the LPA₁ receptor antagonist Ki16425, and LPA increases the expression and release of the profibrotic cytokine CTGF by proximal tubular cells *in vitro*. These observations argue strongly for the involvement of LPA in the development of renal TIF and lead us to propose that the LPA₁ receptor may represent an interesting potential therapeutic target for the treatment renal fibrosis.

RESULTS

UUO-Induced TIF Is Associated with an Increased Release of LPA by Kidney

To determine the possible involvement of LPA in renal TIF, LPA was quantified in conditioned media prepared from kidney explants from mice at different time points after UUO. The

induction of renal TIF was validated by the increase in the level of mRNA encoding two previously characterized TIF and macrophage markers (collagen III and F4/80, respectively) (Figure 1A).¹⁵

LPA was present in conditioned media from kidney explants obtained from nonobstructed kidneys (Figure 1B; time 0). When compared with time 0, LPA concentration in conditioned media was significantly higher at each time point after UUO (3.3-, 3.6-, and 2.9-fold at days 3, 5, and 8, respectively) (Figure 1B). Contralateral kidneys exhibited no significant change in LPA release when compared with time 0 (Figure 1B).

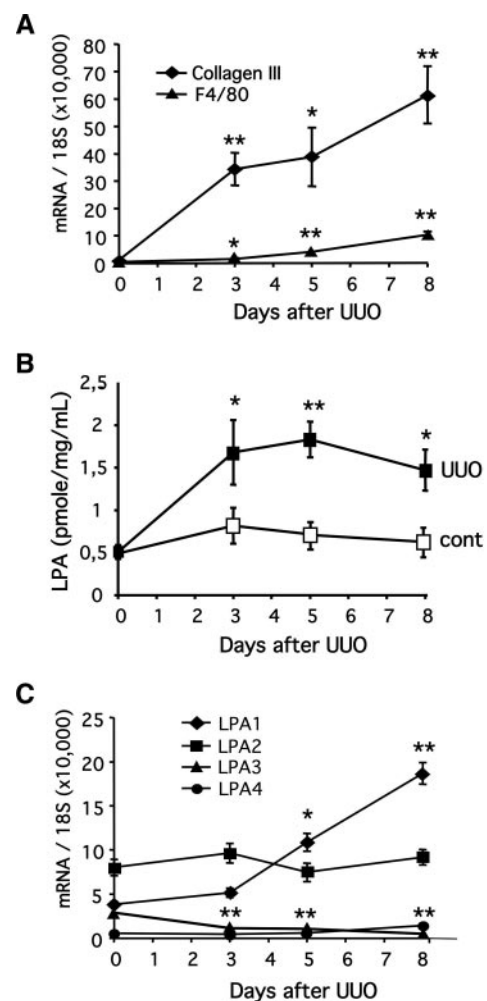


Figure 1. Effect of UUO on the release of LPA and the expression of LPA receptor subtypes in the kidney. Mice were subjected to UUO and kidneys were removed 0, 3, 5, and 8 days after surgery. RNA were extracted from total kidneys and mRNA encoding type III collagen (collagen III) and F4/80 (A) and LPA₁, LPA₂, LPA₃, and LPA₄ receptor subtypes (C) were quantified by real-time PCR. (B) Explants from operated (UUO) and contralateral nonoperated (cont) kidneys were maintained in primary culture for 6 h, and LPA released in the conditioned medium was quantified by a radioenzymatic assay. Values are means \pm SEM from 4 (A through C) and 5 (B) mice for each time point. Comparisons with day 0 were performed using Student *t* test. **P* < 0.05; ***P* < 0.01.

Similarly, sham-operated kidneys exhibited no significant change in LPA release when compared with nonoperated mice (data not shown).

UUO-Induced Renal TIF Is Associated with Upregulation of Renal LPA₁ Receptor Expression

Four LPA receptor subtypes have been identified (LPA₁, LPA₂, LPA₃, and LPA₄).⁶ Real-time reverse-transcription PCR (RT-PCR) analysis revealed that the four subtypes were expressed in total kidney extracts from control mice with the following rank order: LPA₂ > LPA₃ = LPA₁ > LPA₄ (Table 1). Analysis of LPA receptor subtype expression separately in the kidney cortex or in the kidney medulla did not change this expression order (Table 1).

When compared with time 0, the expression of the LPA₁ receptor subtype was significantly increased at day 5 (2.8-fold) and day 8 (4.8-fold) after UUO (Figure 1C). In contrast, the expression of the LPA₃ receptor was significantly decreased at day 3 (4-fold), day 5 (3-fold), and day 8 (4.5-fold) when compared with time 0. No significant change in LPA₂ and LPA₄ receptor expression was observed (Figure 1C). Eight days after surgery, controlateral and sham-operated kidneys exhibited no significant change in gene expression when compared with time 0 (data not shown).

Attenuation of UUO-Induced Renal TIF in LPA₁ Receptor Knockout Mice

The above data suggested that LPA could play a role in UUO-induced renal fibrosis *via* the activation of the LPA₁ receptor. To test this hypothesis, the level of UUO-induced renal TIF was compared between LPA₁(-/-)^{16,17} and LPA₁(+/+) mice. LPA₁(-/-) mice exhibited a slight but nonsignificant reduction in LPA₂ receptor expression when compared with LPA₁(+/+) mice (Table 2). No significant change was observed for LPA₃ receptor mRNA expression. In LPA₁(+/+) mice with a mixed 129SvJ/C57BL/6J background, basal LPA₁ receptor expression was lower than in mice with a pure C57BL/6J background. The LPA₄ receptor was not detectable in mice with the mixed genetic background (Table 2). As shown in Figure 2, mRNA expression of typical fibrosis markers such as collagen type III, α -smooth muscle actin (α SMA), which is a marker of tubulointerstitial myofibroblasts responsible for a large component of collagen deposition in the interstitium, or F4/80 (inflammation) was significantly lower in

Table 1. Expression of LPA-Receptor Subtypes in Kidney^a

LPA Receptor mRNA (/18S RNA × 10,000)	Total	Cortex	Medulla
LPA ₁	3.6 ± 0.5	2.8 ± 0.2	4.9 ± 1.3
LPA ₂	8.1 ± 1.3	6.4 ± 0.7	7.0 ± 1.2
LPA ₃	3.2 ± 0.4	4.4 ± 0.5	3.9 ± 0.6
LPA ₄	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.1

^aValues (mean ± SEM from 4 separate experiments).

Table 2. Expression of LPA-Receptor Subtypes in LPA₁-Knockout and Ki16425-Treated Mice^a

Mice	n	LPA ₁	LPA ₂	LPA ₃	LPA ₄
(+/+)	4	1.3 ± 0.4	4.5 ± 1.8	1.7 ± 0.4	und.
(-/-)	6	und.	1.5 ± 0.29 ns ^b	1.6 ± 0.1 ns	und.
Vehicle	7	3.9 ± 0.8	8.0 ± 1.3	3.5 ± 1.1	0.5 ± 0.1
Ki16425	4	3.4 ± 0.8 ns	9.8 ± 2.1 Ns	7.1 ± 2.1 ns	0.7 ± 0.3 ns

^aValues correspond to mRNA/18S RNA (× 10,000). ns, not significant; und., undetectable.

^bComparisons were performed using Student t test.

LPA₁(-/-) than in LPA₁(+/+) mice. This was confirmed at the protein level for collagen type III and α SMA (Figure 3, A and B). Induction of F4/80 protein tended to be lower in LPA₁(-/-) *versus* LPA₁(+/+) mice, but the difference did not reach statistical significance (Figure 3C).

Attenuation of UUO-Induced Renal TIF by Ki16425 Treatment

Attenuation of UUO-induced TIF in LPA₁(-/-) mice strongly suggested that the LPA₁ receptor was involved in the development of TIF. To strengthen this hypothesis we performed a pharmacological knockout of the LPA₁ receptor by treating obstructed mice with the LPA₁ receptor antagonist Ki16425.^{7,8} In nonobstructed mice, Ki16425 treatment did not significantly change the renal expression of the LPA₁, LPA₂, and LPA₄ receptors when compared with vehicle-treated mice. A slight but nonsignificant increase in LPA₃ receptor expression was observed (Table 2). UUO-induced fibrosis (collagen type III, α SMA) and inflammatory (F4/80) mRNA markers were significantly lower in Ki16425-treated mice than in control mice (Figure 4). This was confirmed at the protein level for F4/80 and collagen type III (Figure 5).

Effect of LPA on CTGF and TGF β Expression In Vivo

CTGF was previously demonstrated to play a crucial role in UUO-induced TIF,^{18,19} and was involved in the profibrotic action of TGF β .⁵ We therefore analyzed TGF β and CTGF mRNA expression in obstructed mice treated with the LPA receptor antagonist Ki16425. We observed that Ki16425 treatment led to a strong attenuation (3- to 4-fold) in the induction of TGF β and CTGF mRNA expression by UUO (Figure 6). These data suggested the involvement of TGF β and CTGF in the profibrotic action of LPA.

Effect of LPA on CTGF and TGF β Expression In Vitro

Finally, we tested whether the profibrotic action of LPA could result from a direct impact of LPA on kidney cells. For that, the mouse epithelial renal cell line MCT was treated with LPA.²⁰ Real-time RT-PCR analysis revealed that MCT cells mainly expressed LPA₁ and LPA₂ receptor subtypes (ratios of 28 ± 7

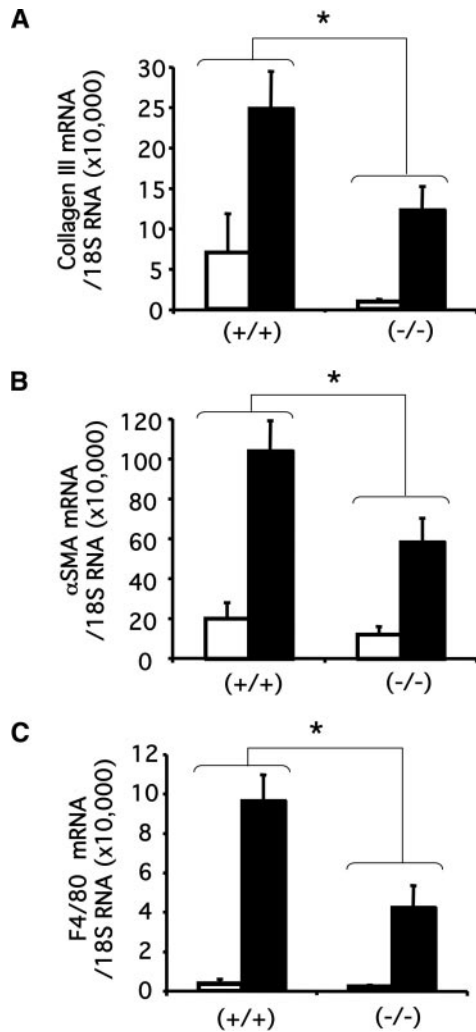


Figure 2. Influence of LPA₁-receptor gene knockout on UUO-induced renal TIF (mRNA expression). LPA₁-receptor knockout mice (-/-) and their wild-type (+/+) littermates were subjected (black bars) or not (white bars) to UUO; kidneys were removed 8 d after surgery. mRNA expression was quantified by real-time PCR: (A) collagen III; (B) α -smooth muscle actin (α SMA); (C) F4/80. Values are means \pm SEM from 6 mice by group. Amplitudes of UUO-induced fibrosis between (+/+) and (-/-) mice were compared by using a two-way ANOVA test. * $P < 0.05$.

and 21 ± 3 to 18S RNA ($\times 10,000$), respectively), whereas LPA₃ and LPA₄ receptor subtypes were undetectable. LPA induced a rapid and transient (Figure 7A) and a dose-dependent (Figure 7B) increase (10-fold maximum) in CTGF mRNA expression. In parallel, LPA exerted only a weak but significant increase (3-fold after 6 h) on TGF β mRNA expression (Figure 7, A and B). CTGF mRNA induction by LPA was almost completely suppressed by cotreatment with the LPA-receptor antagonist Ki16425 (Figure 7C). LPA treatment was also accompanied by a release of CTGF protein in the culture medium of MCT cells, and that release was suppressed by cotreatment with Ki16425 (Figure 7D).

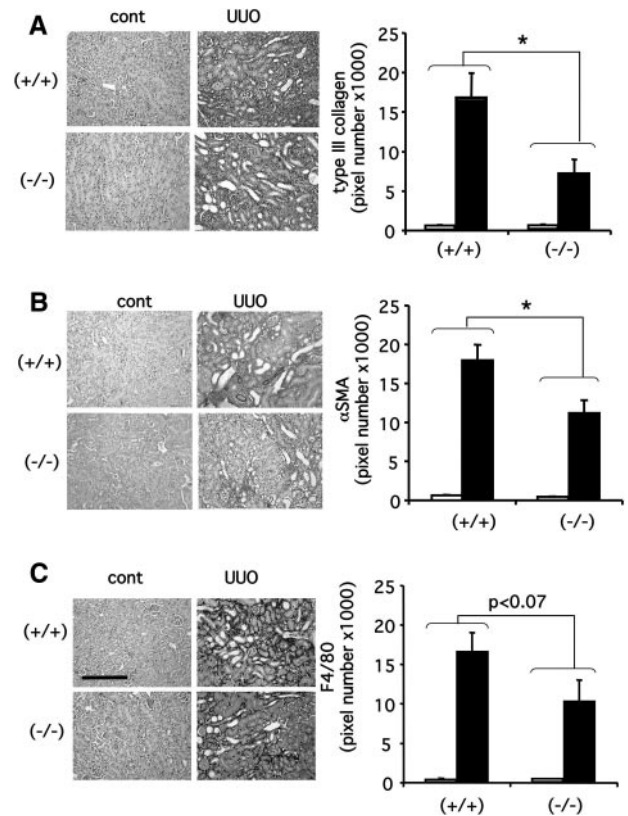


Figure 3. Influence of LPA₁-receptor gene knockout on UUO-induced renal TIF (protein expression). LPA₁-receptor knockout mice (-/-) and their wild-type (+/+) littermates were subjected to UUO (black bars) or not (white bars). Kidneys were removed 8 d after surgery, and protein expression was analyzed with immunohistochemistry: (A) type III collagen; (B) α SMA; (C) F4/80. Representative photographs are shown on the left. Quantification of the photographs is shown on the right. Values are means \pm SEM of 6 mice by group. Amplitudes of UUO-induced fibrosis between (+/+) and (-/-) mice were compared by two-way ANOVA test. * $P < 0.05$. Calibration bar, 250 μ m.

DISCUSSION

This study shows that (1) UUO-induced renal TIF is accompanied by an increased release of LPA, and by an upregulation of LPA₁ receptor expression in the obstructed kidneys; (2) UUO-induced fibrosis is significantly attenuated in kidneys from LPA₁ (-/-) mice as well as in mice treated with the LPA₁ receptor antagonist Ki16425; and (3) on renal proximal tubular cells *in vitro*, LPA increases the expression and release of the profibrotic cytokine CTGF. These observations strongly argue for the involvement of LPA in the development of renal TIF and lead us to propose that the LPA₁ receptor may represent an interesting pharmaceutical target for the treatment of chronic renal disease.

The metabolic origin of LPA released by the kidney, as well as the mechanisms by which the release of LPA is increased after UUO, remain unknown. Several enzymes, including phospholipases A1/A2, lysophospholipase D/autotaxin, glyco-

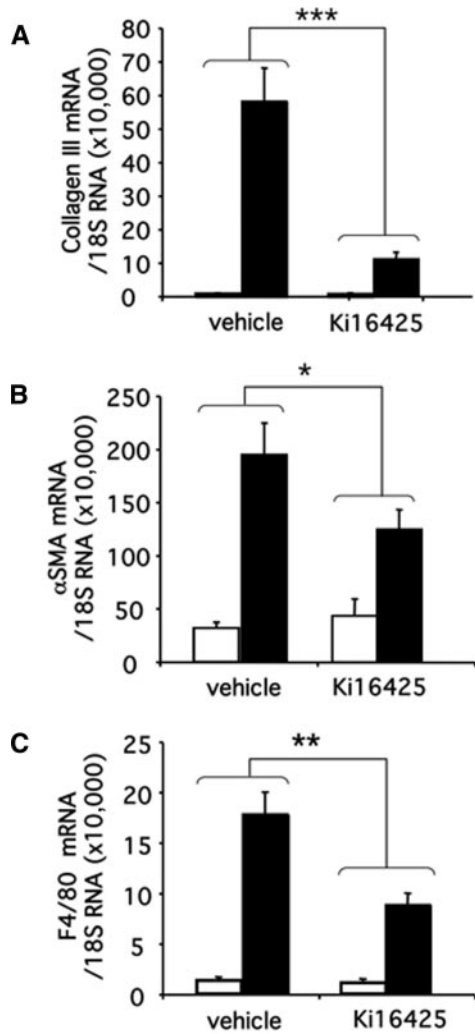


Figure 4. Effect of Ki16425 treatment on UUO-induced renal TIF (mRNA expression). Mice were subjected to UUO (black bars) or not (white bars) in combination with a daily injection of Ki16425 or its vehicle. Kidneys were removed 8 d after surgery, and mRNA expression was determined by real-time PCR: (A) type III collagen; (B) α SMA; (C) F4/80. Values are means \pm SEM from 6 mice by group. Amplitudes of UUO-induced fibrosis between vehicle- and Ki16425-treated animals were compared by two-way ANOVA test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

erol-phosphate acyltransferase, or monoacylglycerol kinase, can possibly lead to renal synthesis of LPA.²¹ Expression and/or the activity of one of these enzymes might be increased in the kidney as an adaptive response to chronic kidney injury induced by UUO. In rat, UUO was shown to increase the activity of a phosphoethanolamine-specific phospholipase A2.²² The involvement of this enzyme in LPA synthesis in the obstructed kidney remains to be explored.

LPA is a growth factor-like phospholipid known to regulate several cellular processes *via* the activation of specific G-protein-coupled receptors (LPA₁, LPA₂, LPA₃, and LPA₄).⁶ We observed that UUO significantly upregulated LPA₁ receptor expression, which suggests that this subtype may play an im-

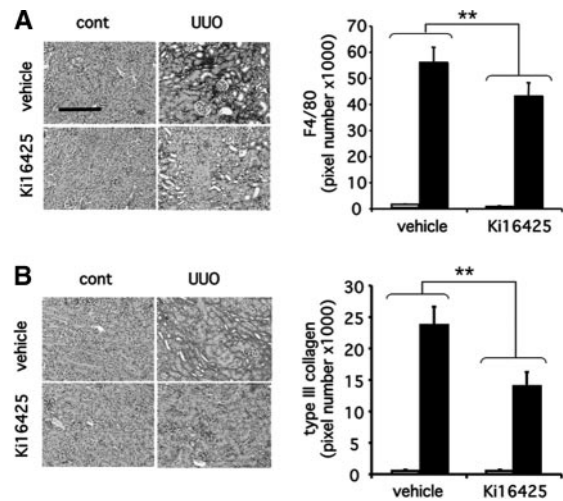


Figure 5. Effect of Ki16425 treatment on UUO-induced renal TIF (protein expression). Mice were subjected to UUO (black bars) or not (white bars) in combination with a daily injection of Ki16425 or its vehicle. Kidneys were removed 8 d after surgery, and protein expression was analyzed by immunohistochemistry: (A) F4/80; (B) type III collagen. Representative photographs are shown on the left. Quantification of the photographs is shown on the right. Values are means \pm SEM from 6 mice by group. Amplitudes of UUO-induced fibrosis between vehicle- and Ki16425-treated animals were compared by two-way ANOVA test. * $P < 0.05$; ** $P < 0.01$. Calibration bar, 250 μ m.

portant role in UUO-induced fibrosis. This hypothesis is supported by our results showing that UUO-induced TIF is significantly attenuated in LPA₁ receptor knockout mice, as well as in mice treated with the LPA₁ receptor antagonist Ki16425. Nevertheless, we found that kidneys also express LPA₂ and LPA₃ receptor subtypes, confirming previous reports,^{11,23} and that UUO reduced LPA₃ receptor expression. Therefore, the involvement of LPA₂ and LPA₃ receptor subtypes in the action of LPA in the development of renal TIF cannot be excluded. In the future, the development of specific LPA₂ or LPA₃ receptor antagonists may help address that hypothesis.

Currently it is not known which renal cells are specifically targeted by LPA and which cells are involved in the LPA₁ receptor-mediated renal fibrosis in ureteral obstruction. The development of renal TIF in UUO is associated with infiltration of inflammatory cells, transformation of epithelial cells into myofibroblasts, proliferation of (myo)fibroblasts, tubular atrophy, and secretion of ECM. On the basis of the literature, LPA can potentially regulate some of these events. LPA has, for example, been demonstrated to participate in intraperitoneal accumulation of monocytes/macrophages^{24,25} as well as in the control of the proliferation of nonrenal myofibroblasts²⁶ and mesangial cells *via* the activation of the ras/MAPK pathway.²⁷ On the basis of our results and previous reports,^{11,23} the expression of the LPA₁ receptor is not different between renal cortex and medulla, suggesting that this receptor subtype is ubiquitously expressed throughout the different areas of the kidney. Consequently, the kidney cell type that is preferentially in-

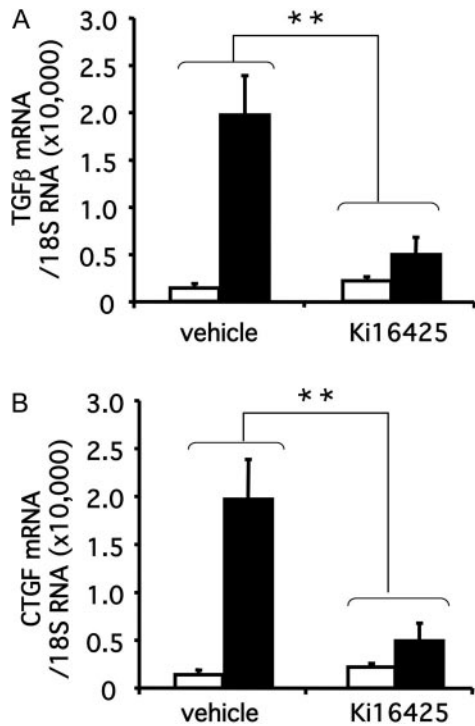


Figure 6. Effect of Ki16425 treatment on UUO-induced renal TIF: Expression of profibrotic cytokines. Mice were subjected to UUO (black bars) or not (white bars) in combination with a daily injection of Ki16425 or its vehicle. Kidneys were removed at day 8 after surgery, and mRNA expression was determined by real-time PCR: (A) TGF β ; (B) CTGF. Values are means \pm SEM from 6 mice by group. Amplitudes of UUO-induced fibrosis between vehicle- and Ki16425-treated animals were compared by two-way ANOVA test. $**P < 0.01$.

involved in the profibrotic activity of LPA remains to be defined. However, on the basis of the observation that UUO-induced fibrosis is essentially interstitial, without visible glomerular lesions,^{14,28} the glomerular LPA₁ receptor is most likely not involved in the effects of LPA on UUO-induced TIF. The remaining cell types that can be potential targets of LPA in the development of UUO-induced renal fibrosis therefore include tubular and inflammatory cells and interstitial fibroblasts. Because LPA was already known to participate in intraperitoneal accumulation of monocytes/macrophages^{24,25} and that LPA can induce expression of the profibrotic cytokine CTGF in primary culture human fibroblasts,²⁹ we focused the remainder of our studies on the *in vitro* effects of LPA treatment on tubular cells. In addition, it has been shown that primary culture human proximal tubular cells express the LPA₁ receptor.³⁰

Among the UUO-induced factors that are strongly attenuated by LPA₁ receptor blockade is the profibrogenic factor CTGF. Interestingly, we found that LPA was able to upregulate CTGF expression and secretion in cultured proximal tubular cells. Similar observations were made previously in renal fibroblasts and mesangial cells.^{29,31,32} Our results show that the action of LPA on CTGF expression is very likely mediated by the

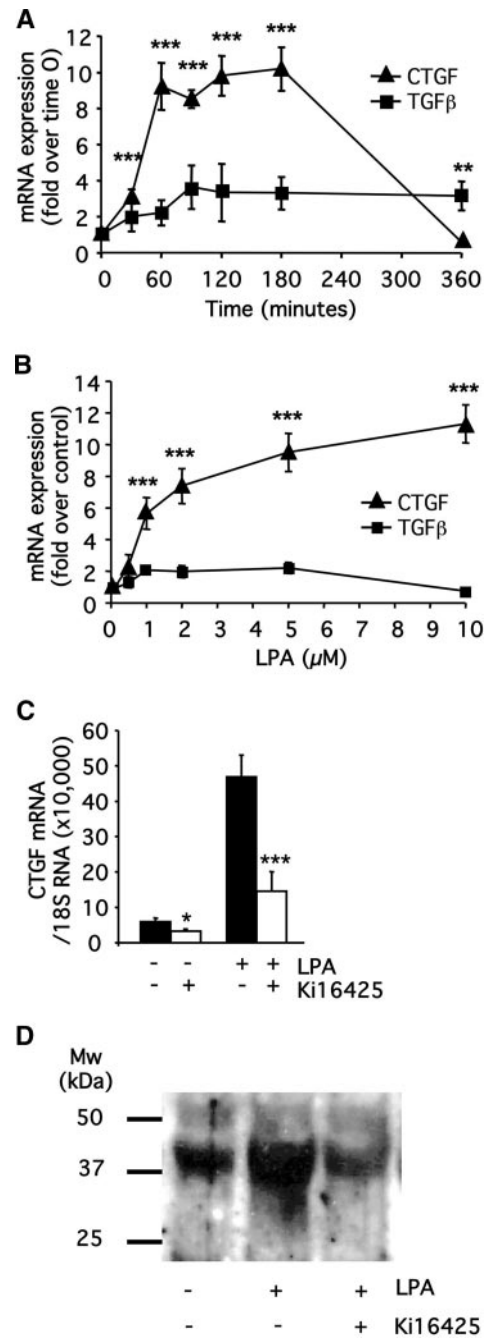


Figure 7. Effect of LPA on CTGF expression in MCT cells. CTGF and TGF β mRNA were quantified in serum-starved MCT cells exposed to 2 μ M LPA for increasing time (A) or to increasing concentrations of LPA for 2 h (B); $***P < 0.001$ when compared with time 0 (A) or to the absence of LPA (B) (determined by *t* test). (C) CTGF mRNA were quantified in serum-starved MCT cells exposed to 2 μ M LPA \pm 10 μ M Ki16425: $*P < 0.05$; $**P < 0.01$ when compared with LPA alone (determined by *t* test). Values are means \pm SEM from 3 separate experiments. (D) Serum-starved MCT cells were exposed to 2 μ M LPA \pm 10 μ M Ki16425, and the release of CTGF protein in the culture medium for 3 h was analyzed by Western blot (representative of 2 separate experiments).

LPA₁ receptor subtype because Ki16425 blocks these effects. Consequently, the parallel between *in vivo* and *in vitro* experiments suggests that the profibrogenic effect of LPA could in part be mediated by increased CTGF expression and secretion. CTGF induction by LPA in mesangial cells was shown to be mediated by the small GTPase rhoA and the downstream kinase ROCK.³¹ Interestingly, treatments with ROCK inhibitors have been described to attenuate UUO-induced renal TIF,³³ similar to what we observed in LPA₁(-/-) and in Ki16425-treated mice.

The *in vivo* expression of the profibrogenic factor TGFβ is also significantly attenuated by LPA₁ receptor blockade. In contrast to CTGF, *in vitro* LPA treatment of MCT cells only modestly modified TGFβ expression. This difference suggests that regulation of TGFβ and CTGF expression and secretion by LPA involves different transduction pathways and/or can occur in different kidney cell types.

Therefore, combining our studies and the published data on the effects of LPA on renal CTGF and TGFβ production, the antifibrotic effect of LPA₁ receptor blockade can potentially involve three cell types with important roles in the development of UUO-induced TIF: inflammatory cells, tubular cells, and fibroblasts.

In conclusion, our study demonstrates for the first time, using both genetically engineered animals and pharmacological tools, that LPA and its LPA₁ receptor could play an important fibrotic role in UUO-induced TIF *via* a mechanism involving in part the profibrotic cytokine CTGF. Because TGFβ has many other effects,³⁴ its blockage is not a realistic therapeutic option to reduce renal fibrosis. On the other hand targeting the CTGF has been shown as a promising antifibrotic therapy.¹⁹ Therefore, pharmacological inhibition of LPA synthesis or antagonizing LPA₁ receptors might be interesting in the treatment of renal fibrosis.

CONCISE METHODS

Animals

Male LPA₁(-/-) mice and their wild-type (WT) littermates were on a mixed 129SvJ/C57BL/6J background.^{16,17} For all other experiments, C57BL/6J mice were used (Harlan, Gannat, France). Mice were handled in accordance with the principles and guidelines established by the National Institute of Medical Research (INSERM). They were housed in a pathogen-free animal facility with constant temperature (20 to 22°C), humidity (50 to 60%), and with a 12-h/12-h light/dark cycle (lights on at 8:00 a.m.). All mice had free access to food (energy contents in % kcal: 20% protein, 60% carbohydrate, and 20% fat; (Usine d'Alimentation Rationnelle, Villemoisson-sur-Orge, France) and water throughout the experiment.

UUO

Mice (8 wk old) were used in these experiments. UUO was performed as described previously.¹⁵ Mice were euthanized at different time points (0, 3, 5, and 8 d) after UUO, and the kidneys were dissected for

further analysis. Control kidneys were dissected from nonoperated mice. All experiments reported were conducted in accordance with the principles and guide lines established by INSERM and were approved by a local animal care and use committee.

Treatment with Ki16425

Ki16425 (Sigma, Saint Quentin Fallavier, France) powder was first diluted in DMSO at the concentration of 100 μg/μl and then in PBS at the final concentration of 5 μg/μl. Male C57BL/6J mice were injected subcutaneously with the Ki16425 solution at the dose of 20 mg/kg per d or with the vehicle (100 μl injection volume). Injections began 1 d before UUO surgery and were repeated daily for 8 d.

Culture of Kidney Explants

Explants were prepared from the distal pole of the kidneys. Explants (9 to 30 mg) were incubated at 37°C in 12 wells per plate containing 1 ml serum-free DMEM supplemented with 1% BSA (≥97% free fatty acids; Sigma) for 6 h in a humidified atmosphere containing 7% CO₂. After incubation, conditioned media were separated from explants, centrifuged to eliminate cell debris, and frozen at -20°C for further analysis.

LPA Quantification

LPA was extracted from conditioned media and quantified by radioenzymatic assay as described previously.³⁵

mRNA Quantification

Total RNAs were extracted using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany). Gene expression was analyzed using real-time RT-PCR as described previously.³⁶ Oligonucleotides for mouse gene expression studies were:

LPA₁ receptor—sense: 5'-CATGGTGGCAATCTACGTCAA-3'; antisense: 5'-AGGCCAATCCAGCGAAGAA-3'

LPA₂ receptor—sense: 5'-TGTCTGACTGCACAGCTTGGGA-3'; antisense: 5'-CTCATGGAGTTTTCTGGTGCC-3'

LPA₃ receptor—sense: 5'-GTACCTGAGCCCCCATTTG-3'; antisense: 5'-AAACCCATGCGGAAACAAC-3'

LPA₄ receptor (also known as p2y9/GPR23)—sense: 5'-CCT-TACCAACATCTATGGGAGCAT-3'; antisense: 5'-TGGCCAG-GAAACGATCCA-3'

F4/80—sense: 5'-TGACAACCAGACGGCTTGTG-3'; antisense: 5'-GCAGGCGAGGAAAAGATAGTGT-3'

Collagen type III—sense: 5'-ACGTAGATGAATTGGGATG-CAG-3'; antisense: 5'-GGGTTGGGGCAGTCTAGTC-3'

αSMA—sense: 5'-GTCCAGACATCAGGGAGTAA-3'; antisense: 5'-TCGGATACTTCAGCGTCAGGA-3'

CTGF—sense: 5'-GGCATCTCCACCCGAGTTAC-3'; antisense: 5'-GATTTTAGGTGTCCGGATGCA-3'

TGFβ—sense: 5'-GAGCCCGAAGCGGACTACTA-3'; antisense: 5'-CACTGCTTCCCGAATGTCTGA-3'

Immunohistochemistry

Immunohistological staining and analysis of kidney sections were performed as described previously.¹⁵ Rat monoclonal antibody to mouse F4/80 (RM2900; Caltag Laboratories Inc., Burlingame, CA)

was used for macrophage detection. Collagen type III and α -SMA were detected using rabbit anti-human collagen type III (T59105R Interchim, Montluçon, France) and the monoclonal mouse anti-human α -SMA (DAKO EPOS method, U7033; DAKO S.A., Trappes, France), respectively. For the visualization of collagen type III, the DAKO Envision System was used (DAKO S.A.). For all samples, negative controls for the immunohistochemical procedures included substitution of the primary antibody with nonimmune sera.

Histomorphometric Analysis

As described previously,¹⁵ an operator unaware of the origin of each kidney section performed analyses. Under a light microscope (Nikon Eclipse 600, Tokyo, Japan) at $\times 200$ magnification, 10 nonoverlapping fields (to obtain approximately 80% of the kidney section) per kidney section were captured with an analogic camera (MicroFire CCD color; Optronics, Goleta, CA) connected to the microscope. Quantitative analysis of the pictures was performed with Adobe PhotoShop 5.5 software (Adobe Systems Incorporated, San Jose, CA), which allows counting of the pixels stained specifically (brown for the immunohistochemical studies).

Culture of MCT Cells and Preparation of Conditioned Media

MCT cells were a kind gift of Dr M. Zeisberg (Harvard Medical School, Cambridge, MA). Cells were grown until confluence in DMEM supplemented with 5% fetal calf serum. MCT cells were washed twice with PBS to remove serum and then incubated (4 ml for a 10-cm diameter plate) in serum-free DMEM supplemented for 3 h with or without pharmacological reagents. Conditioned medium was collected and centrifuged to eliminate cell debris, and concentrated (about 50 fold) using an Amicon Ultra 10,000 (Millipore) and stored at -20°C before analysis.

Detection of CTGF Secretion by Western Blot

Concentrated conditioned medium (50 μg) were loaded and separated on a Gel Nu-PAGE (Invitrogen, Cergy Pointoise, France) 4-20% and transferred on nitrocellulose membrane. The blot was incubated overnight at 4°C in TBS/Tween 0.1% containing 5% BSA and then for 1 h at room temperature in the same solution supplemented with 0.4 $\mu\text{g}/\text{ml}$ CTGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After washing in TBS/Tween 0.1%, CTGF was visualized by enhanced chemoluminescence detection system using an anti-rabbit-horseradish peroxidase antibody.

Statistical Analysis

Values are means \pm SEM. The interaction of UUO-induced fibrosis with LPA₁ knockout or Ki16425 treatment was statistically analyzed by a multivariate analysis (two-way ANOVA). Other comparisons were performed with a *t* test. Differences were considered significant at $P < 0.05$.

ACKNOWLEDGMENTS

This work was supported by grants from INSERM and the National Institutes of Health (MH51699 and NS048478). J.K. was supported by

a grant from the Ministère de l'Éducation Nationale de la Recherche et de la Technologie (France). We thank Y. Barreira and C. Nevoit (IFR31 Animal Facility), J.J. Maoret (IFR31 Molecular Biology Platform) for technological assistance. We would like to thank Dr M. Zeisberg for his generous gift of MCT cells.

DISCLOSURES

None.

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