Uric acid increases fibronectin synthesis through upregulation of lysyl oxidase expression in rat renal tubular epithelial cells

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Yang Z, Xiaohua W, Lei J, Ruoyun T, Mingxia X, Weichun H, Li F, Ping W, Junwei Y. Uric acid increases fibronectin synthesis through upregulation of lysyl oxidase expression in rat renal tubular epithelial cells. Am J Physiol Renal Physiol 299: F336–F346, 2010. First published May 19, 2010; doi:10.1152/ajprenal.00053.2010.—Urate is produced as the major end product of purine metabolism. In the last decade, the incidence of hyperuricemia increased markedly, and similar trends in the epidemiology of metabolic syndrome have been observed. Hyperuricemia is associated with renal disease, and recent studies have reported that mild hyperuricemia results in hypertension, intrarenal vascular disease, and renal injury. This has led to the hypothesis that uric acid may contribute to renal fibrosis and progressive renal disease. Our purpose was to investigate the relationship between uric acid and renal tubular injury. We applied the method of intraperitoneal injection of uric acid to generate the hyperuricemic mouse model. Compared with the saline injection group, the expression of lysyl oxidase (LOX) and fibronectin in kidneys was increased significantly in hyperuricemic groups. In vitro, uric acid significantly induced NRK-52E cells to express the ECM marker fibronectin, as well as LOX, which plays a pivotal role in ECM maturation, in a time- and dose-dependent manner. Upregulation of the urate transporter URAT1, which is located in the apical membrane of proximal tubules, sensitized the uric acid-induced fibronectin and LOX induction, while both knocking down URAT1 expression in tubular epithelial cells by RNA interference and inhibiting URAT1 function pharmacologically attenuated LOX and fibronectin expression. Furthermore, knockdown of LOX expression by a small interfering RNA strategy led to a decrease in fibronectin abundance induced by uric acid treatment. In addition, evidence of a uric acid-induced activation of the NF-κB signaling cascade was obtained both in primary cultured renal proximal tubular epithelial cells. Our findings highlight a need for carefully reevaluating our previous view on the pathological roles of hyperuricemia in the kidney and nephropathy induced by uric acid in clinical practice.

NF-κB

URATE, WHICH IS A COLORLESS, tasteless, weak organic acid, is the final breakdown product of unwanted purines in higher primates, as they lack the enzyme uricase that, in other species, converts urate into allantoin. The biosynthesis of urate is catalyzed by xanthine oxidase and/or its isoinom, xanthine dehydrogenase. Approximately two-thirds of the daily turnover of urate is accounted for by urinary excretion, with the remaining one-third being excreted into the gut as feces. In the human kidney, urate is reabsorbed and secreted via a widely predicted in the renal proximal tubules (16). Urate is transported via its transporters across the apical membrane of proximal tubular cells, in exchange for anions being transported back into the tubule lumen to maintain electrical balance. Urate then moves across the basolateral membrane into the blood by another transporter. The recently identified human urate transporters included URAT1 (1), SLC2A9 (35), MRP4 (34), OAT1, and OAT3 (13), among which URAT1 is thought to be the major mechanism for regulating blood urate levels. URAT1 is expressed in the apical membrane of proximal tubule cells by the SLC22A12 gene, and mutation of the gene causes idiopathic renal hypouricemia (9, 21).

Although it was reported that urate has beneficial effects of scavenging potential harmful radicals in our body (31), it can also cause significant health problems in conjunction with certain genetic or environmental factors, including complications associated with urate crystals such as kidney stones and gout (33). There is also increasing evidence that persons with elevated uric acid may be at increased risk for cardiovascular and renal disease (6, 15, 23, 38). Hyperuricemia seems to be a cofactor in hypertension, a marker and possibly itself responsible for microvascular damage through stimulation of the renin-angiotensin system, inhibition of endothelial nitric oxide, and proliferative effects on vascular smooth muscle (2, 8, 19, 20). Hyperuricemia may also play a role in metabolic syndrome, triggering insulin resistance and hypertension (12, 25). Uric acid may mediate aspects of the relationship between hypertension and kidney disease via renal vasoconstriction and systemic hypertension (7). An elevated serum uric acid level is a modest, independent risk factor for new-onset kidney disease in the general population (38). Recent studies have reported that mild hyperuricemia in normal rats results in hypertension, intrarenal vascular disease, and renal injury (20). Evidence of a uric acid-induced activation of NF-κB signaling cascade was observed. Our findings highlight a need for carefully reevaluating our previous view on the pathological roles of hyperuricemia in the kidney and nephropathy induced by uric acid in clinical practice.

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globular proteins with pI > 8.0 could be potential substrates of LOX. It has been reported that the proteolytically activated pre-LOX can form a bound complex with cellular fibronectin at the surface of human fibroblasts in vivo (11). Additional experiments in this study demonstrated tight complexes formed in vitro between purified bovine LOX and fibronectin that retained catalytic function. Ectopic pre-LOX expression inhibited fibronectin-stimulated protein tyrosine phosphorylation, while the mature LOX enzyme enhanced fibronectin-stimulated integrin signaling (41). It suggested that there might be intricate interactions between LOX and fibronectin, which largely remain unclear.

To examine the effect of hyperuricemia on renal disease progression in vivo, mice were daily injected intraperitoneally with 250 mg/kg uric acid for various time periods. Serum uric acid level, histological sections of kidney, Western blots, and immunofluorescence were carefully examined. In vitro, we focused on the effects of uric acid on renal proximal tubular epithelial cells in the production of fibronectin, a phenotypic marker for ECM. Our findings suggest that uric acid could increase synthesis of fibronectin via URAT1 through upregulation of LOX expression.

**MATERIALS AND METHODS**

**Cell culture and treatment.** Rat proximal tubular epithelial cells (NRK-52E) were kindly provided by the Cell Resource Center at the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY). For uric acid treatment, NRK-52E cells were seeded at 80% confluence in complete medium containing 10% fetal bovine serum. After 24 h, the cells were changed to serum-free medium and incubated for an additional 16 h. Then, uric acid (Sigma) was added in different concentrations for various periods of time as scheduled. The cells were then collected at different time points for further characterization. The pharmacological inhibitor probenecid was obtained from Sigma (P8761, Sigma-Aldrich), and the NF-κB inhibitor BAY 11-7082 was obtained from Beyotime (S1523, Beyotime).

**Animal model.** Male BALB/c mice, weighing 21–23 g, were acquired from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences. They were housed in the animal facility at the Experimental Animal Center of Nanjing Medical University with free access to food and water. Animals were treated humanely, using approved procedures in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institutes of Health at Nanjing Medical University. Animals were randomly assigned into three groups (with 5 mice/group): the sham-operated group, hyperuricemia for 7 days group, and hyperuricemia for 28 days group. The mouse hyperuricemia model was prepared at 5-8°C for protein extraction.

**Western blot analysis.** NRK-52E cells and uric acid-treated cells were lysed with SDS sample buffer (62.5 mM Tris·HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue). Kidney tissues were homogenized by a polytron homogenizer (Brinkmann Instruments, Westbury, NY) in RIPA lysis buffer (1% NP-40, 0.1% SDS, 100 μg/ml PMSF, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 2 μg/ml aprotinin, and 2 μg/ml leupeptin in PBS) on ice. The supernatants were collected after centrifugation at 13,000 g at 4°C for 20 min. Protein concentration was determined using a BCA protein assay kit (Sigma), and whole-tissue lysates were mixed with an equal amount of 2× SDS loading buffer (125 mM Tris·HCl, 4% SDS, 20% glycerol, 100 mM DTT, and 0.2% bromophenol blue). Samples were heated at 100°C for ~5–10 min before loading and were separated on precast 10 or 5% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA). Detection of protein expression by Western blotting was carried out according to the established protocols described previously (40). The primary antibodies used were as follows: anti-fibronectin (F-3648, Sigma-Aldrich), anti-LOX (sc-32409, Santa Cruz Biochemical), anti-actin (A5385, Sigma-Aldrich), anti-LOX (sc-32409, Santa Cruz Biochemical), anti-phospho-NF-κB p65 (Ser32; 93H1, no. 3033, Cell Signaling), anti-NF-κB p65 (no. 3034, Cell Signaling), anti-phospho-IκB-α (Ser32; 14D4; no. 2859, Cell Signaling), anti-IκB-α (4D4; no. 4812, Cell Signaling), anti-phospho-IKKα (Ser180; no. 2681, Cell Signaling), and anti-IKKβ (no. 2682, Cell Signaling). Quantification was performed by measurement of the intensity of the signals with the aid of the National Institutes of Health Image software package.

**RT-PCR analysis.** Total RNA was prepared using a TRIzol RNA isolation system according to the instructions specified by the manufacturer (Invitrogen). The first strand of cDNA was synthesized using 2 μg of RNA in 20 μl of reaction buffer using Moloney leukemia virus-RT (Promega, Madison, WI) and random primers at 42°C for 30 min. PCR was performed using a standard PCR kit on 1-μl aliquots of cDNA and HotStarTaq polymerase (Promega) with specific primer pairs. The sequences of primer pairs were as follows: fibronectin (forward) 5′-GTG GCT GCC TTC AAC TTT CTC-3′ and (reverse) 5′-AGT CCT TTA GGG CCG TCA AT-3′; LOX (forward) 5′-CTA TGA CAC TTA GGA GAG ACC GGT CCG GG-3′ and (reverse) 5′-CAG AGC GTA CGA CAT TGT TAC TGT AGT CTG-3′; URAT1(forward) 5′-GTC TT CTT CTT CCA TCA AGC-3′ and (reverse) 5′-CAA ACA GGT ATG GCC AGG TAC TC-3′; and β-actin (forward) 5′-CAG CTG AGA GGA AAA TCG TG-3′ and (reverse) 5′-CTG TCG TAA CAA TAG TGA TGA CC-3′. The PCR products were size fractionated on a 1.0% agarose gel and detected by NA-green (D0133, Beyotime) staining.

**Immunofluorescence staining.** Indirect immunofluorescence staining was performed according to an established procedure (39). Briefly, cells cultured on coverslips were washed twice with cold PBS and fixed with cold methanol/acetone (1:1) for 10 min at ~20°C. Following three extensive washings with PBS, the cells were blocked with 0.1% Triton X-100 and 2% normal donkey serum in PBS buffer for 40 min at room temperature and then incubated with the specific primary antibodies described above, followed by staining with FITC- or TRITC-conjugated secondary antibody. Kidney cryosections were prepared at 5-μm thickness and fixed for 10 min with cold methanol/acetone (1:1). After being blocked with 20% normal donkey serum in PBS for 40 min, the sections were incubated with anti-fibronectin or anti-LOX antibody. As a negative control, the primary antibody was substituted with nonimmune IgG and no staining occurred. Cells and the kidney cryosections were double stained with 4′,6-diamidino-2-phenylindole to visualize the nuclei. Renal proximal tubules were stained with lectin from Tetragonolobus purpureas. Slides were viewed with a Nikon Eclipse 80i Epi-fluorescence microscope equipped with a digital camera (DS-R1, Nikon). In each experimental setting, immunofluorescence images were captured with identical light exposure times.

**Morphological studies.** Tissue sections from the mice were prepared at 5-μm thickness by a routine procedure. Sections were stained with hematoxylin/eosin for general histology.

**LOX or URAT1 knockdown by specific small interfering RNA.** Small interfering (si) RNA and negative control siRNA (acquired from Ambion) were transfected into cells using Lipofectamine 2000 reagent (Invitrogen) according to the protocols provided by the manufacturer. Briefly, 24 h before transfection, NRK-52E cells were plated in 2 ml of growth medium without antibiotics so that the cells would be ~50% confluent at the time of transfection. For each
transfection sample, the preparation of siRNA:Lipofectamine 2000 complexes was as follows. Dilution of 100 pmol of siRNA in 250 μl of Opti-MEM I Reduced Serum Medium without serum (GIBCO) with gentle agitation was done. Five microliters of Lipofectamine 2000 was diluted in 250 μl of Opti-MEM I Medium with gentle agitation, and then incubated for 5 min at room temperature. After the incubation, the diluted siRNA and Lipofectamine 2000 were combined (total volume 250 μl). After brief and gentle agitation, the mixture was incubated for 20 min at room temperature to allow the siRNA: Lipofectamine 2000 complexes to form. Two hundred and fifty microliters of siRNA:Lipofectamine 2000 complexes were added to each well plated with NRK-52E cells previously and then incubated at 37°C in a CO2 incubator for 48 h until the cells were ready for gene knockdown assay.

Plasmid transfection. URAT1 expression plasmid was kindly provided by 301 Hospital of the PLA (Beijing, China). The empty expression plasmid vector pcDNA3 was purchased from Invitrogen (San Diego, CA). Plasmids were transfected into cells using Lipofectamine 2000 transfection reagent (Invitrogen) according to the protocols provided by the manufacturer. Briefly, 24 h before transfection, NRK-52E cells were plated in 2 ml of growth medium without antibiotics to make up ~90% confluent at the time of transfection. The preparation of transfection samples was completed as follows. Plasmid:Lipofectamine 2000 complexes were made by diluting 0.2, 1, or 4 μl of plasmid in 250 μl of Opti-MEM I Reduced Serum Medium without serum (GIBCO), and then 0.5, 2.5, or 10 μl Lipofectamine 2000 were added to 250 μl of Opti-MEM I Medium. After gentle mixing and incubating for 5 min at room temperature, the diluted plasmid and Lipofectamine 2000 (total volume ~510 μl) were mixed together followed by 20-min incubation at room temperature with gentle agitation. The complexes were added to each culture well with a slight rocking motion of the plate. Incubation of the cells at 37°C in a CO2 incubator for 24 h was carried out until the cells were ready for gene transfection assay.

Statistical analysis. Animals were randomly assigned to control and experimental groups. Western blotting, RT-PCR, and immunofluorescence staining were all performed at least three times independently. Statistical analysis was performed using SigmaStat software (Jandel Scientific Software, San Rafael, CA). Comparisons between groups were made using one-way ANOVA, followed by Student’s t-test. P < 0.05 was considered significant.

RESULTS

Uric acid induces fibronectin synthesis in vitro. To demonstrate the synthesis of ECM by tubular epithelial cells in vitro, we examined the de novo expression of fibronectin, a phenotypic marker for ECM, in rat renal tubular epithelial cells (NRK-52E). As shown in Fig. 1A, incubation of NRK-52E cells with uric acid induced abundant expression of fibronectin protein. Dose-dependent studies revealed that uric acid was able to induce fibronectin at a concentration as low as 60 μmol/l, suggesting that the induction could be readily achievable in vivo under pathological conditions. As shown in Fig. 1, B and D, the induction of de novo expression of fibronectin reached significant magnitude after uric acid treatment followed by 12 h of incubation. Figure 1, E and F, shows the deposition of fibronectin of tubular epithelial cells after uric acid treatment. The presence of abundant fibronectin-positive microfilaments in the intercellular domain is evident.

Upregulation of URAT1 sensitizes tubular epithelial cell expression of fibronectin in response to uric acid treatment. Urate excretion and reabsorption require specialized transporters located in renal proximal tubule cells. URAT1, one of the recently identified human urate transporters, has been considered to play important roles in homeostasis and might sensitize...
Uric acid-induced renal tubular cell injury. With respect to this hypothesis, we examined the effects of the upregulation of URAT1, which increased uric acid being transported into tubular cells, on fibronectin expression in tubular epithelial cells. As shown in Fig. 2A, Western blot analysis reveals that upregulation of URAT1 induced fibronectin protein expression in NRK-52E cells. Under the same conditions, no fibronectin protein induction was found in NRK-52E cells transfected with the empty vector. Of note, the magnitude of fibronectin induction in URAT1-expressing cells was comparable to that stimulated by uric acid. This suggests that uric acid-initiated fibronectin induction was partly mediated by URAT1. Forced expression of URAT1 in tubular epithelial cells sensitized uric acid-induced fibronectin induction in a plasmid dose-dependent manner (Fig. 2, B and D).

Knockdown of URAT1 expression in tubular epithelial cells by RNA interference attenuates fibronectin expression. To investigate the impacts of downregulation of the uric acid being transported into NRK-52E cells on fibronectin expression, we used a siRNA approach to knock down URAT1 expression in NRK-52E cells. This posttranscriptional gene-silencing strategy has been demonstrated to knock down specific gene expression by using sequence-specific siRNA. Therefore, three double-stranded siRNAs corresponding to three different regions of rat URAT1 cDNA were transiently transfected into NRK-52E cells. Figure 3A shows the results of URAT1 levels in NRK-52E cells by RT-PCR analysis. Quantitative determination revealed that the URAT1 mRNA level was reduced by ∼90% (URAT1-808), 70% (URAT1-954), and 20% (URAT1-1018), which is in accordance with the mRNA levels of URAT1 and fibronectin (Fig. 3A). Under the same conditions, expression of unrelated genes such as actin was unaltered.

Fig. 2. Upregulation of urate transporter URAT1 induces FN expression. A: NRK-52E cells were transfected with empty vector pcDNA3 or URAT1 expression vectors. After transfection, cells were treated with 80 μmol/l of uric acid for 24 h. Cell lysates were immunoblotted with antibodies against FN. B: Western blot analysis shows that forced expression of URAT1 sensitized uric acid (80 μmol/l)-induced FN in a pURAT1 dose-dependent manner. C and D: graphic presentation of the relative abundance of FN (arbitrary units) in plasmid-transfected NRK-52E cells incubated with uric acid. *P < 0.01 vs. uric acid-incubated cells transfected with pcDNA3 (n = 3).
protein expression in a time-dependent manner. Western blot analyses revealed that endogenous pre-LOX and mature LOX started to increase as early as 6 and 12 h after treatment, respectively. This induction of LOX by uric acid in tubular epithelial cells was also dose dependent (Fig. 5C). Maximal induction of mature LOX was found at 180 μmol/l of uric acid.

Interference of urate transporter URAT1 affects LOX expression in vitro. As mentioned above, URAT1 expression and function influence the expression of fibronectin induced by uric acid. We then examined its impact on the expression of LOX. Figure 6A shows that forced expression of URAT1 by plasmid transfection into tubular epithelial cells sensitized uric acid-induced pre-LOX induction in a plasmid dose-dependent manner. Pharmacologically, inhibition of the function of the urate transporter by probenecid decreases the expression of pre-LOX protein in a probenecid dose-dependent manner (Fig. 6B). Meanwhile, knockdown of URAT1 expression in tubular epithelial cells by RNAi attenuates pre- and mature LOX expression. As in Fig. 6, C and D, RT-PCR and Western blot analysis show the results of LOX mRNA, pre-, and mature LOX protein levels in NRK-52E cells. Compared with cells transfected with control siRNA, quantitative determination revealed that the LOX protein levels in cells transfected with URAT1 siRNA after uric acid treatment were reduced by ~80 (URAT1-808) and 90% (URAT1-954) compared with cells transfected with negative control siRNA, which is in accordance with the mRNA levels of URAT1 (Fig. 3A) and LOX (Fig. 6C). As indicated above, siRNA URAT1-1018 is not as efficient as URAT1-808 and -954 in URAT1 knockdowns. Neither the mRNA nor the protein levels of LOX were significantly reduced after siRNA URAT1-1018 transfection with or without uric acid treatment. Under the same conditions, expression of unrelated genes such as actin was unaltered.

Knockdown of LOX attenuates fibronectin expression. As stated above, the cellular consequences of uric acid treatment is the de novo production of fibronectin, a marker of ECM. Along this line, we examined the effects of LOX on fibronectin expression in LOX-knockdown tubular epithelial cells. Again, the RNAi approach was adopted to knock down LOX expression in NRK-52E cells. Double-stranded siRNA was transiently transfected into NRK-52E cells. As shown in Fig. 7, A and B, RT-PCR and Western blot analysis reveal that the expression of LOX mRNA and protein was downregulated both before and after incubation with uric acid compared with NRK-52E cells transfected with negative control siRNA. Meanwhile, Fig. 7C shows the results of fibronectin levels in NRK-52E cells by Western blot analysis. Compared with cells transfected with control siRNA, quantitative determination revealed that the fibronectin protein levels of cells transfected with LOX siRNA after uric acid treatment were reduced by ~80–90%. Under the same conditions, expression of unrelated genes such as actin was unaltered.

Fig. 3. Knockdown of URAT1 by RNA interference (RNAi) attenuates FN expression. A: RT-PCR analysis demonstrates URAT1 and FN mRNA levels after transient transfection of URAT1 small interfering (si) RNA without or with uric acid (180 μmol/l) treatment. β-Actin was amplified simultaneously from the exact cDNA to ensure equal loading of each lane. B: Western blot analysis demonstrates FN protein level after transient transfection of URAT1 siRNA without or with uric acid treatment. C: graphic presentation of the relative abundance of FN protein after transient transfection of URAT1 siRNA without or with uric acid treatment. *P < 0.01 vs. control (n = 3), **P < 0.01 vs. uric acid-incubated cells transfected with negative control siRNA (n = 3).

Fig. 4. Blockage of transporter of uric acid by probenecid decreases FN synthesis. A: Western blot analysis demonstrates the expression of FN protein level after pharmacological inhibition of urate transporter by probenecid without or with uric acid (180 μmol/l) treatment. B: graphic presentation of the relative abundance of FN in NRK-52E cells incubated with uric acid after probenecid treatment. *P < 0.01 vs. control (n = 3), **P < 0.01 vs. uric acid-incubated cells without probenecid treatment (n = 3).
URIC ACID INCREASES FN SYNTHESIS BY LOX

F341

Fig. 5. Induction of lysyl oxidase (LOX) is observed during uric acid treatment. A and B: NRK-52E cells were treated with uric acid (180 μmol/l) in serum-free medium for various periods of time as indicated. A: time course of pre-LOX expression. B: time course of mature LOX expression. C: NRK-52E cells were treated without (control) or with different amounts of uric acid for 24 h in serum-free medium. Western blot analysis demonstrates the dose course of mature LOX expression.

genes such as actin was unaltered, suggesting the specificity of LOX inhibition by the RNAi strategy. Figure 7, D–G, shows the deposition of fibronectin after uric acid treatment of tubular epithelial cells transfected with control or LOX siRNA. Compared with cells transfected with control siRNA, immunofluorescence staining revealed that the uric acid-induced deposition of fibronectin protein of cells was significantly attenuated after transfection with LOX siRNA.

Induction of LOX and fibronectin during ECM assembly in hyperuricemic mouse kidney. To demonstrate tubular epithelial cell injury in vivo under pathological conditions, we examined the expression of LOX and fibronectin during the progression of ECM assembly in the hyperuricemic mouse kidney. Figure 8 shows the change in serum uric acid level (Fig. 8A) and morphological injury (Fig. 8, B–D) in hyperuricemic mice following intraperitoneal injection of either uric acid or normal saline. Renal injury, characterized by tubular dilatation with epithelial atrophy, interstitial expansion, and matrix deposition, occurred in the early stage after hyperuricemia and was progressive in a time-dependent manner (Fig. 8, B–D). Furthermore, we examined the expression and deposition of LOX and fibronectin. Figure 8, E–L, shows the results of LOX and fibronectin deposition in hyperuricemic kidneys as determined by Western blotting and immunofluorescence staining. As expected, both in Western blot analysis of whole kidney lysates (Fig. 8, E and F) and immunofluorescence staining (Fig. 8, G–L), the expression of pre-LOX and fibronectin was markedly and progressively increased in the kidneys following intraperitoneal injection of uric acid.

Activation and expression of NF-κB signaling pathway in uric acid-induced kidney injury in vitro. Because uric acid has been reported to activate NF-κB in other cell types, the possible involvement of NF-κB in uric acid-induced renal tubular cell injury was nonetheless examined further. The activated form of NF-κB is composed of two proteins, p65 (Rel A) and p50 subunits. In the classic pathway, NF-κB is bound and inhibited by IκB proteins. Proinflammatory cytokines, growth factors, or antigen receptors activate an IKK complex (IKKα, IKKβ, and NEMO), which phosphorylates IκB proteins. Phosphorylation of IκB leads to its ubiquitination and proteasomol degradation, freeing NF-κB. Active NF-κB is further activated by phosphorylation and translocation to the nucleus, where alone or in combination with other transcription factor families, they induce target gene expression. We examined the activation status and expression level of NF-κB proteins in NRK-52E cells incubated with uric acid for various time periods. As shown in Fig. 9A, NF-κB was markedly phosphorylated in a very early stage after uric acid treatment, as demonstrated by Western blot analysis using a phospho-specific antibody. The phosphorylated NF-κB p65 was increased as early as 5 min after incubation with uric acid and was sustained during the first hour, and then decreased in the second hour. However, the total NF-κB p65 abundance was not altered at different time points tested. Similar results were obtained for phospho-IκB-α (Fig. 9B) and phospho-IKKe (Fig. 9C) expression.

To further evaluate the involvement of NF-κB in mediating the uric acid-induced expression of LOX and fibronectin, the effect of NF-κB inhibitor BAY 11-7082 (functionally acting as an inhibitor of IκB-α phosphorylation, NF-κB p65 phosphorylation, and nuclear translocation) on uric acid-induced LOX and fibronectin synthesis was examined. Figure 9E shows that the NF-κB blocker significantly reduced the effect of uric acid on LOX and fibronectin protein synthesis in NRK-52E cells. In addition, BAY 11-7082 blocked uric acid-induced phosphorylation of NF-κB p65 (Fig. 9D).

DISCUSSION

In the last decade, both hyperuricemia and gout have increased markedly and similar trends in the epidemiology of metabolic syndrome have been observed (27). This study presents evidence that uric acid induces ECM synthesis in both hyperuricemic mouse kidneys and cultured rat renal proximal tubular epithelial cells. The serum uric acid level of a normal Sprague-Dawley rat is ~50 μmol/l; accordingly, we chose to use the dose of 60–240 μmol/l in our in vitro study. As shown in Figs. 1A and 5C, the induction of fibronectin and LOX expression by uric acid in vitro was dose dependent, and the maximal induction was found at 180 μmol/l of uric acid treatment. We therefore chose 180 μmol/l in most of our cell experiments. As to the animals, the mean serum uric acid level of sham-operated mice was 58.3 μmol/l as we measured. The mean serum uric acid level tripled after 7 days of injection, and increased nearly five times 28 days later (Fig. 8). Our studies suggested that the effect of uric acid on fibronectin synthesis is probably mediated via URAT1 through upregulation of LOX expression.
In humans, urate is the final breakdown product of unwanted purines. Hyperuricemia may occur due to decreased excretion of uric acid (underexcretion), increased production (overproduction), or a combination of these two mechanisms. In the human kidney, filtered urate is mainly reabsorbed in the proximal tubules (28). The reabsorption and secretion processes depend on specific transporter molecules that reside in the membranes of proximal cells. Based on membrane vesicle studies, there are two transporters, a voltage-sensitive pathway and an urate/anion exchanger. The known urate transporters in the human kidney are URAT1 (SLC22A12), MRP4, OAT1, and OAT3. URAT1 is expressed exclusively in the apical membrane of proximal tubule cells of humans, mice, and rats, through which up to 90% of the daily load of urate filtered by the kidney is reabsorbed into the proximal tubule cells. URAT1 is presumably absent in mammals predominantly secreting urate.

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Fig. 6. Interference of urate transporter URAT1 affects LOX expression in vitro. A: upregulation of URAT1 by plasmid transfection induces pre-LOX protein expression in cells incubated with 80 μmol/l of uric acid in a URAT1 dose-dependent manner. B: pharmacological inhibition of urate transporter by probenecid decreases uric acid (180 μmol/l)-induced pre-LOX protein synthesis. C and D: knockdown of URAT1 by RNAi attenuates LOX expression without or with uric acid (180 μmol/l) treatment. Shown are RT-PCR analysis of downregulated LOX mRNA expression after URAT1 siRNA transfection (C) and Western blot analysis of downregulated pre- and mature LOX protein expression after siRNA transfection (D).

Fig. 7. Knockdown of LOX by RNAi attenuates FN expression. A: RT-PCR analysis demonstrates LOX mRNA levels after transient transfection of LOX siRNA without or with uric acid (180 μmol/l) treatment. B and C: Western blot analysis demonstrates the mature LOX (B) and FN (C) protein levels after transient transfection of LOX siRNA without or with uric acid (120 and 240 μmol/l) treatment. D–G: immunofluorescence staining for FN in NRK-52E cells after control or LOX siRNA transfection without or with uric acid (180 μmol/l) treatment. D: control siRNA. E: control siRNA plus uric acid (180 μmol/l). F: LOX siRNA. G: LOX siRNA plus uric acid.
urate, such as rabbits and pigs. However, in humans, urate secretion is probably negligible and URAT1 is thought to be the major mechanism for regulating blood urate levels. Consistent with this, mutations of URAT1 cause idiopathic renal hypouricemia, which is a rare disorder with a prevalence of 0.12% (9, 18). The lack of functional URAT1 results in lower blood levels of urate and high urinary urate levels, resulting in crystal formation within the kidney tubules.

Hyperuricosuria, which may result from hyperuricemia, can be a cause of stone formation. It was reported that up to 20% of patients with hyperuricemia developed urate stones (16). It may be due to either excessive dietary intake of purine-rich food or endogenous overproduction of urate. Additionally, when there is massive tissue breakdown, especially resulting from chemotherapy or radiation therapy, which is termed “tumor lysis syndrome,” there may be a substantial release of DNA and RNA, resulting in a large purine load, followed by a marked rise in serum urate level and urinary urate concentration. This increased urate level results in intratubular crystallization with obstruction and acute renal failure. Local inflam-
mation and interstitial fibrosis may be the consequences if the obstruction is prolonged. In addition, elevated levels of serum uric acid in patients with gout could trigger a local inflammatory reaction, neutrophil recruitment, and the production of proinflammatory cytokines as well as other inflammatory mediators (4, 36, 37). However, the virtual pathophysiological changes in the hyperuricemic kidney seem to be different from gouty nephropathy. Unfortunately, previous studies have not paid much attention to or even neglected the differences between hyperuricemic nephropathy and gouty nephropathy. The underlying mechanism of hyperuricemia-induced renal injury, especially the pathoepoiiesis of uric acid in renal proximal tubule cells, needs further investigation. An understanding of the effects of uric acid on renal proximal tubule cells is important, as they are the major sites for uric acid reabsorption and secretion in the kidney.

LOX has long been the subject of considerable interest as the catalyst of the posttranslational oxidation of peptidyl lysine to the peptidyl aldehyde. Since the first demonstration of its activity in vitro in 1968, LOX has been recognized as the catalyst that accounts for stabilization of extracellular collagen and elastin. The insolubilization of the soluble precursors of ECM proteins to insoluble fibers results from the intra- and interpeptide chain cross-linkage generated as the product of LOX action on these structural macromolecules (22). Recent investigations demonstrated that the biological role of LOX has already extended beyond the oxidation of ECM proteins. Indeed, several reports describe its influence on cell proliferation, intracellular signal responses, cell migration, and tissue development and reveal that it may act as an antagonist or a protagonist of malignant processes. It has also been shown that this enzyme can reenter cells and then concentrate within cell nuclei (5, 10, 17, 24, 26, 29, 30, 32). Despite LOX’s multifunctionality, we focused on its classic role on the ECM in this study. Our data suggest that uric acid may exert its profibrotic effects through upregulation of LOX. This notion is based on the observations that uric acid increased fibronectin protein in cultured rat renal proximal tubule cells in a LOX-dependent manner and RNA interference of LOX attenuated the effects of uric acid on ECM formation in vitro, which was associated with a substantial reduction in fibronectin protein synthesis. Moreover, we found that LOX was significantly increased in fibronectin deposited in the kidneys of hyperuricemic mice, which substantiates the notion that lysyl oxidase may play a role in the development and progression of hyperuricemia-induced renal injury. Although initially identified by their ability to cross-link ECM protein, LOX has also been shown to carry out intracellular functions and display a extended range of biological activities including the regulation of ras and NF-κB signaling (24). Recent reports indicate that LOX interaction with the transcriptional repressor Snail induces renal fibrosis (3). It is plausible that a uric acid-induced increase in the expression of lysyl oxidase in tubular epithelial cells enables promotion of ECM assembly. However, the molecular events underlying ECM cross-linkage are complex, and because of the recently limited understanding of LOX protein maturation, localization, and function, its role in fibronectin synthesis via modulation of other molecular pathways, such as the Notch or TGF-β signaling pathways, awaits further investigation.

The mechanisms by which urate crystals elicit an inflammatory response in gout joints have begun to be unraveled (4).
The urate crystals evoke an inflammatory infiltrate rich in neutrophils in animal and patient gout models. The capacity of urate crystals to stimulate monocyte/macrophages as well as tissue-resident cells, such as synoviocytes, to release IL-1β was recognized several years ago. The binding between urate crystals, Toll-like receptors, and CD14, located on the surface of monocytes, promotes cell activation of the NF-κB signaling pathway through MYD88-dependent mechanisms, leading to caspase-1 processing of pro-IL-1β. Meanwhile, tissue-resident cells are stimulated to produce IL-1β and other cytokines, such as TNF-α, IL-6, MIP-2, etc., resulting in a proinflammatory response. Our studies show that in rat renal proximal tubule cells, uric acid was observed to activate NF-κB, resulting in a phosphorylation of NF-κB, IkB, and IKK. Our observation of activation of the NF-κB in response to uric acid is in agreement with previous reports with mononuclear phagocytes, smooth muscle cells, and rabbit proximal tubule epithelial cells (14), in which NF-κB activation by uric acid resulted in a stimulation of cell proliferation or growth inhibition. Meanwhile, our studies indicated that inhibition of NF-κB attenuated uric acid-induced LOX and fibronectin expression. However, we have not been able to determine the mediator through which uric acid activated the NF-κB signaling cascade. Further investigation will be necessary to unravel the relevance of these findings to the onset of renal disease.

In summary, our studies suggest that uric acid increases ECM synthesis both in vivo and in vitro via urate transporters through upregulation of lysyl oxidase expression. The NF-κB signaling pathway is involved in the hyperuricemia-induced proximal tubule injury. Our findings highlight a need for carefully reevaluating our previous view on the pathological roles of hyperuricemia in the kidney and nephropathy induced by uric acid in clinical practice.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


