Soluble uric acid increases NALP3 inflammasome and interleukin-1β expression in human primary renal proximal tubule epithelial cells through the Toll-like receptor 4-mediated pathway

JING XIAO*, XIAO-LI ZHANG*, CHENSHENG FU, RUI HAN, WEIJUN CHEN, YIJUN LU* and ZHIBIN YE

Department of Nephrology, Huadong Hospital Affiliated to Fudan University, Shanghai 200040, P.R. China

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Abstract. Urate crystals activate innate immunity through Toll like receptor 4 (TLR4) activation, leading to the formation of the NACHT, LRR and PYD domains-containing protein 3 (NALP3; also known as NOD-like receptor family, pyrin domain containing 3 (NALP3) and cryopyrin) inflammasome, caspase-1 activation and interleukin (IL)-1β expression in gout. However, whether elevated serum uric acid (UA) levels are associated with the development and progression of renal diseases without renal urate crystal deposition remains unknown. In the present study, human primary renal proximal tubule epithelial cells were incubated with soluble UA (100 µg/mL) with or without the TLR4 inhibitor, TAK242 (1 µM). The gene expression and protein synthesis of TLR4, NALP3, caspase-1, IL-1β and intercellular adhesion molecule-1 (ICAM-1) were detected by real-time PCR, ELISA, western blot analysis and fluorescence-activated cell sorting (FACS), respectively. Soluble UA significantly enhanced TLR4, NALP3, caspase-1, IL-1β and ICAM-1 expression in the human primary renal proximal tubule epithelial cells. The TLR4 inhibitor, TAK242 effectively blocked the soluble UA-induced upregulation of TLR4, NALP3, caspase-1, IL-1β and ICAM-1 expression in the human primary renal proximal tubule epithelial cells. Our findings indicate that soluble UA enhances NALP3 expression, caspase-1 activation, IL-1β and ICAM-1 production in renal proximal tubule epithelial cells in a TLR4-dependent manner, suggesting the activation of innate immunity in human primary renal proximal tubule epithelial cells by soluble UA.

Introduction

Hyperuricemia is a consistent and independent risk factor for hypertension, metabolic syndrome, fatty liver, diabetes and kidney diseases (1-3). Tubulointerstitial inflammation and injury are commonly detected in hyperuricemia-induced chronic renal injury, with increased macrophage and T cell infiltration observed in the tubulointerstitium of the kidneys (4,5). As some patients with hyperuricemia present with urate crystal deposition in the tubules and interstitium, it was previously assumed that hyperuricemia causes tubular injury through the precipitation of urate in the form of crystals in the kidneys, in a manner similar to which it causes gout. In gout, urate crystals cause inflammation by stimulating leukocytes to produce the pro-inflammatory cytokine, interleukin-1β (IL-1β). IL-1 production occurs when the urate particles are ingested by phagocytes through the Toll like receptor 4 (TLR4) activation pathway, which facilitates the formation of the NACHT, LRR and PYD domains-containing protein 3 (NALP3; also known as NOD-like receptor family, pyrin domain containing 3 (NALP3) and cryopyrin) inflammasome, thus promoting pro-caspase-1 activation, further activating caspase-1; activated caspase-1 then cleaves pro-IL-1β into its active form IL-1β and, thus, initiates downstream inflammatory processes (6,7). Accumulating evidence has demonstrated that urate crystal-induced inflammation is a paradigm of innate immunity. Innate immunity-related components, including TLR4, NALP3, ASC, caspase-1 and IL-1β are essential in the development of gouty inflammation (8). However, recent findings suggest that the presence of elevated soluble serum uric acid (UA) levels represent the presence of low-grade systemic inflammation even in the absence of gout (9). A recent ultrasound study also suggested that ultrasonographic changes suggestive of gouty arthritis in joints and tendons may occur in patients with asymptomatic hyperuricaemia who have never had an episode of gout (10). Moreover, as urate crystal is as less likely to be deposited in the kidneys as in the joints, these recent findings suggest that, not only urate crystals, but also soluble UA may account for the injury in patients with hyperuricemia. However, whether soluble UA can also lead to the TLR4-mediated activation of innate immunity remains unknown.

Soluble UA may have direct pro-inflammatory effects as shown by previous studies, including stimulating the production of C-reactive protein (CRP) and monocyte chemotactic...
protein-1 (MCP-1) in vascular cells through the activation of nuclear factor-xB (NF-xB) and mitogen-activated protein kinase (MAPK) (11,12), inducing endothelial dysfunction with mitochondrial alterations and decreased intracellular adenosine triphosphate (ATP) concentrations (13), exerting pro-oxidative effects mediated in part by the activation of the lactate dehydrogenase reductase (NADPH) oxidase system and stimulating mitochondrial oxidative stress in vascular cells or adipocytes (14). In the kidneys, observational studies have also observed renal injuries induced by soluble UA without urate crystal formation induced by inflammation (15-18). In clinical observations, not only hyperuricemia, but also a higher normal level of UA has been shown to increase the risk of the early progression of renal function loss in patients with type 1 diabetes (19). In hyperuricemic rats, renal disease has been shown to progress rapidly without the presence of urate crystals in the kidneys (18). In vitro findings have also suggested that soluble UA actively participates in pro-inflammatory processes in vascular smooth muscle cells and mesangial cells (11,15,16). However, these studies failed to illustrate the mechanisms of soluble UA-induced renal injury and did not investigate the upstream events of the pro-inflammatory effects of soluble UA.

As kidney diseases, including some metabolic renal injuries, often manifest with renal immune dysregulation (20), it is possible that soluble UA may directly induce renal tubular injury without urate crystal deposition. More importantly, the TLR4-mediated activation of innate immunity may be involved as an upstream event for the pro-inflammatory effects of soluble UA. Therefore, we hypothesized that soluble UA may cause innate immune injury through the TLR4-dependent pathway, and may thus promote the formation of the NALP3 inflammasome, the activation of caspase-1, the expression of IL-1β and the overproduction of downstream inflammatory cytokines. In order to verify this hypothesis, in the present study, we incubated human primary renal proximal tubule epithelial cells (PTECs) with soluble UA and then measured the expression levels of TLR4, the NALP3 inflammasome, caspase-1 and IL-1β, as well as those of the inflammatory marker, intercellular adhesion molecule-1 (ICAM-1). We also used the TLR4-specific inhibitor, TAK242, to block the effects of TLR4 so as to determine whether the effects of soluble UA on PTECs are TLR4-dependent.

Materials and methods

Reagents. Human primary renal PTECs and their culture medium were purchased from ScienCell (San Diego, CA, USA). UA was purchased from Sigma (St. Louis, MO, USA). The TLR4 inhibitor, TAK242, was purchased from Chembest Research Laboratories Ltd. (Shanghai, China). The MTT assay kit was from Amresco (Solon, OH, USA). Reagents for real-time PCR were purchased from Takara (Kyoto, Japan). Enzyme immunoassay kits for the detection of IL-1β were purchased from eBioscience (San Diego, CA, USA). Anti-TLR4 (Cat. no. ab22048) and anti-ICAS/NALP3 (Cat. no. ab16097) antibodies were from Abcam (Cambridge, UK). Anti-caspase-1 (Cat. no. sc-515) and anti-ICAM-1 (Cat. no. sc-8439) antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-mouse (Cat. no. A0216) and anti-rabbit (Cat. no. A0208) secondary antibodies were from SinoBios (Shanghai, China).

Cell culture. Human primary renal PTECs were cultured in epithelial cell medium, which contains 500 ml of basal medium, 50 ml of fetal bovine serum, 5 ml of epithelial cell growth supplement and 5 ml of penicillin/streptomycin solution. The cells were incubated at 37°C in 5% CO₂ and 95% air. In all the experiments, there was a ‘growth arrest’ period of 24 h in serum-free medium prior to stimulation.

Preparation of soluble UA. UA was dissolved in 1 M NaOH at a concentration of 50 mg/ml as previously described (21). The solution was examined free of mycoplasma and filtered (22 μm pore size) before use. Crystals were not detectable (polarizing microscopy), nor did they develop during cell incubation.

Viability of PTECs under increasing concentrations of soluble UA. To examine cell viability following treatment with various concentrations of soluble UA, the growth-arrested human primary renal PTECs were seeded in 96-well plates (0.25x10⁵ cells/well) and exposed to soluble UA (0-800 μg/ml) for 24, 48 and 72 h, respectively. The effects of treatment with TAK242 on cell viability (0.25-2 μM) were also examined. Subsequently, the cytotoxic effects of these stimulations on the human primary renal PTECs were examined by MTT assay. Briefly, 20 μl MTT solution were added to each well, and the cells were incubated at 37°C in 5% CO₂ and 95% air for 4 h. The reaction was terminated by the addition of 150 μl DMSO, and the absorbance was measured at 570 nm by an ELISA reader. All results were expressed as percentage changes in absorbance compared with those of the medium control (defined as human primary renal PTECs incubated with plain culture medium).

Total RNA extraction and real-time PCR for the quantification of TLR4 and ICAM-1 gene expression. The growth-arrested human primary renal PTECs were incubated 100 μg/ml of soluble UA for 4 h. Total cellular RNA was extracted using the NucleoSpin RNA II total RNA extraction kit. The quality of the extracted RNA was monitored by formaldehyde agarose gel electrophoresis. Aliquots of each RNA extraction were reverse transcribed simultaneously into cDNA using the OneStep RT-PCR kit (Takara, Tokyo, Japan) according to the manufacturer’s instructions. Each real-time PCR reaction was performed in a total volume of 25 μl in duplicate using the SYBR® Premix Ex Taq™ kit (Takara, Kyoto, Japan) and the Fast Real-Time PCR system 7500 (Applied Biosystems Inc., Foster City, CA, USA). Primers and probe sets for human TLR4 and ICAM-1 were designed from known sequences in GenBank and the primer sequences are listed in Table I. GAPDH was used as an endogenous control to normalize the amount of cDNA added to each reaction (ΔC_t), and the mean ΔC_t value of the control samples was used as the calibrator to calculate the ΔΔC_t value. The quantification of each transcript was calculated using the comparative Ct method. In this method, the relative quantity of the target mRNA, normalized to the endogenous control and relative to the calibrator, is equal to 2−ΔΔC_t.

ELISA of IL-1β protein synthesis in cell culture supernatants. The growth-arrested human primary renal PTECs were incubated with 100 μg/ml soluble UA for 48 h. Cell culture supernatants were collected and stored at -70°C until use in the protein assay. The protein levels of IL-1β in the culture superna-
The protein expression of TLR4, the NALP3 inflammasome and caspase-1.

After harvesting the cell culture supernatant as described above, the remaining cells were lysed with lysis buffer containing protease inhibitor cocktail (Sigma). Ten micrograms of total protein extracted from $10^6$ cells were electrophoresed through a 12% SDS-PAGE gel before being transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking for 1 h at room temperature in blocking buffer [5% bovine serum albumin in Tris-buffered saline (TBS) with 0.05% Tween-20 (TBST)], the membranes were incubated overnight with mouse anti-TLR4 (1:500), mouse anti-CIAS1/NALP3 (1:1,000), rabbit anti-caspase-1 p10 (1:500) and rabbit anti-GAPDH (1:10,000) in TBST. The membranes were washed and incubated for 1 h at room temperature with a peroxidase-labeled goat anti-rabbit (Cat. no. A0208) or goat anti-mouse (Cat. no. A0216) immunoglobulin (SinoBios). After further washing, the membranes were detected by ECL chemiluminescence (Amersham Pharmacia Biotech, Arlington Heights, IL, USA).

ICAM-1 detection by fluorescence-activated cell sorting (FACS) analysis. After an incubation of 48 h with 100 µg/ml of soluble UA, the human primary renal PTECs were detached with 0.25% trypsin in 1 mM ethylenediamine tetraacetic acid-4Na, washed twice with PBS buffer, pH 7.2, and then incubated with fluorescein isothiocyanate-conjugated anti-human ICAM-1 (1:50; Santa Cruz Biotechnology) for 1 h in an ice-bath. The expression of ICAM-1 was detected using a FACSScan (BD FACS Calibur; BD Bioscience, San Jose, CA, USA). Data were compared as the geometric mean (GMean) values of the fluorescence intensity of the ICAM-1-positive cells. The same experiment was repeated 3 times.

Statistical analysis. All data are expressed as the means ± SD unless otherwise specified. Statistical analysis was performed using SPSS v.19.0 for Windows (SPSS, Inc., Chicago, IL, USA). Intergroup differences for continuous variables were assessed by multivariate ANOVA. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Viability of human primary renal PTECs and pH value of the medium following incubation with soluble UA and TAK242. The viability of the human primary renal PTECs cultured with serial dilutions of soluble UA and TAK242 (TLR4 inhibitor) for 24, 48 and 72 h was examined by MTT assay. The results revealed that soluble UA (Fig. 1A) from 0 to 800 µg/ml and (B) TAK242 from 0.25 to 2 µM did not affect the cell viability at all the time points examined. Results are expressed as percentage changes in absorbance relative to those of the human primary renal PTECs incubated in plain medium alone. All results represent the means ± SD obtained from 3 independent experiments.
Soluble UA increases TLR4 expression in human primary renal PTECs. To determine whether the innate immunity of the human primary renal PTECs by soluble UA was activated, the expression of TLR4, a typical membrane receptor for innate immunity (22), was examined following treatment with 100 μg/ml soluble UA for 4 h (gene expression) or 48 h (protein expression). The TLR4-specific inhibitor, TAK242 (1 μM), was added to the human primary renal PTECs 1 h prior to stimulation with soluble UA. The results from real-time PCR and western blot analysis revealed that soluble UA increased the (A) mRNA expression and (B) protein synthesis of TLR4. TAK242 significantly blocked the 100 μg/ml soluble UA-induced increase in (A) TLR4 mRNA expression and (B) TLR4 protein synthesis. All results represent the means ± SD obtained from 3 independent experiments. **P<0.01 vs. human primary renal PTECs cultured in plain medium; #P<0.01 vs. human primary renal PTECs cultured with the same concentration of soluble UA but without TAK242. Representative images of the corresponding protein bands are shown at the top of each bar in (B).

Induction of TLR4-dependent ICAM-1 expression by soluble UA in human primary renal PTECs. To determine whether the activation of TLR4 is specific for soluble UA-induced ICAM-1 expression, the TLR4-specific inhibitor, TAK242 (1 μM), was added to the human primary renal PTECs 1 h prior to stimulation with soluble UA. Soluble UA significantly promoted ICAM-1 expression in the human primary renal PTECs (Fig. 3B; P<0.05), whereas TAK242 reversed this soluble UA-induced activation of caspase-1 in the human primary renal PTECs (Fig. 3B; P<0.05).

Discussion
Serum UA levels are elevated in chronic kidney disease and closely correlate with the progression of renal disease (26). However, whether soluble UA actively participates in renal...
injury is incompletely understood, which substantially limits our clinical understanding of this recently revisited disease at the entity. In the present study, we found that soluble UA significantly induced the upregulation of pro-inflammatory cytokine ICAM-1 expression with TLR4-NALP3-caspase-1-IL-1β signaling pathway activation in cultured human primary renal proximal tubule epithelial cells (PTECs), indicating that soluble UA may participate in the development and progression of renal disease through the TLR4-mediated activation of innate immunity.

Our results are indicative as we selected human primary renal PTECs, which share most of the properties of the human situation compared with other immortalized human renal tubular cells or primary cells from other species. Studies on the effects of UA on tubular cells have mainly used rat (4,27-29) and rabbit (30) tubular cells or the human proximal tubule epithelial cell line, HK-2 (29,31). In the studies on rat renal proximal tubular cells, UA was shown to possess pro-inflammatory and pro-fibrotic properties (4,27,28). One study in 2007 found that UA inhibited rat renal proximal tubule cell proliferation through at least two signaling pathways involving protein kinase C, MAPK, cytosolic phospholipase A2 and NF-kB (30). Later, UA was shown to induce cell apoptosis by regulating apoptotic proteins (29) in the human renal tubular cell line, HK-2, to induce epithelial-to-mesenchymal transition (27) and to increase fibronectin synthesis (28) in rat renal
epithelial cells. These studies have elegantly demonstrated the downstream effects of UA; however, they failed to provide the upstream information for the mechanisms through which UA triggers these pro-inflammatory cascades. In the present study, we observed the upregulation of ICAM-1, an important pro-inflammatory mediator mediating the interaction of resident renal cells to immune cells (32). The soluble UA-induced overexpression of ICAM-1 was dependent on TLR4 activation, as the TLR4 inhibitor reversed this soluble UA-induced upregulation of ICAM-1, suggesting that TLR4-mediated immunity is UA-dependent and may be capable of leading to the activation of other pro-inflammatory cascades.

Innate immunity has recently been found to be of great interest even in several metabolic diseases (20). It is closely associated with disease initiation and progression; the participation of the innate and the adaptive immune response in mechanisms that contribute to inflammation in cardiovascular disease has been reported in atherosclerosis and hypertension (33). The involvement of immune dysregulation in cardiac, vascular and renal changes in hypertension has been demonstrated in experimental models (34). Renal epithelial cells are also immune privileged and are surrounded by a dense network of immune cells, which provides an environment for the communication of tubular cells with immune cells. In fact, renal epithelial cells share many phenotypic and functional characteristics with mononuclear phagocytes, such as the secretion of chemokines in response to direct stimulation with TLR ligands (35) and the expression of major histocompatibility complex (MHC) I and II, as well as co-stimulatory molecules. There are even data suggesting that proximal tubules can present antigen to T cells (36).

The urate crystal form of UA, monosodium urate (MSU) crystals, has been suggested to be a so-called ‘danger signal’ that alerts the immune system to cell injury and helps to trigger both innate and adaptive immune responses (7). The triggered immune responses initiate the production of pro-inflammatory cytokines and then facilitate the communication between immune cells and local tissue cells. It is only found in immune cells and soluble UA directly activates T cells without antigen presentation (21).

In the present study, we provide further evidence in that soluble UA modulates the innate immune injury with the formation of the TLR4-dependent NALP3 inflammasome, resulting in caspase-1 activation, IL-1β and ICAM-1 production in human primary renal PTECs. Innate immunity has been shown to trigger inflammation in a number of diseases (37) and, thus, our findings suggest that the many pro-inflammatory effects of UA may be initiated by the activation of innate immunity through TLR4. Targeting the innate immunity pathway may provide an alternative for the treatment of diseases with inflammation.

TLRs are typical membrane receptors for innate immunity and are the first line of defense during the innate immune response (38). Studies have documented the TLR4-mediated pro-inflammatory effects in podocytes (39), oxidative stress in mesangial cells (40) and renal tubular damage (41,42) in proximal tubular cells under the stimulation of high glucose, lipopolysaccharide and lipids. TLR4 has also been recognized as a receptor for UA and plays a significant role in amplifying inflammatory effects (6). In peripheral blood from patients with acute gout arthritis, the NF-κB level and IL-1β production were markedly reduced after TLR4 blockade with anti-TLR4 antibody (43), suggesting the key role of TLR4 in gout. TLR4 signaling, including the adaptor molecule myeloid differentiation factor 88 (MyD88) and the inflammasome complex, actively participates in mediating experimental tubule-interstitial nephritis (44). In experimental renal transplantation, local TLR4 activation by endogenous ligands may be a pathological link from unspecific primary damage to subsequent chemokine release, and the infiltration and activation of immune cells, leading to the deterioration of renal function and the induction of renal fibrosis, in which UA may play an important role (45). However, to the best of our knowledge, whether TLR4 is an alternative pathway for UA activation in renal tubular cells and whether it is a link between metabolic injury to subsequent inflammatory cytokine release has not been previously examined. In the present study, TLR4 expression was elevated by soluble UA and the TLR4 inhibitor, TAK242, significantly blocked the downstream NALP3 inflammasome, caspase-1 activation, IL-1β and ICAM-1 synthesis induced by soluble UA, indicating the TLR4-dependent effects of soluble UA. The inflammatory cytokine, ICAM-1, was also blocked by the TLR4 inhibitor, suggesting that TLR4 may be such a bridge to link metabolic damage to inflammatory injury.

Extensive examination of the entering of UA into tubular cells has revealed the presence of urate transporters on both sides of tubular cells which mediate the absorption and excretion of UA (46), as well as many of the detrimental effects of
UA (4,27). However, in a previous study, the inhibitor of urate transporter could not completely block the pro-inflammatory effects of UA (28), suggesting the activation of other systems which may also account for the injury. The innate immune response has also been demonstrated to participate in recognition, uptake and the responses of cells to MSU crystals in immune cells. Recognition of the naked MSU crystal by TLR2 and TLR4 has been shown to promote the ingestion of naked MSU crystal by phagocytes (6,47). As the uptake of urate by urate transporters has not been examined for its association with innate immunity, it is possible that TLR4 may also participate in soluble UA uptake and play a role in soluble UA-induced renal injury. Therefore, in the present study, we used 1 µM of the TLR4 inhibitor, TAK242, which has been shown to fully block TLR4 signaling, in order to examine the specific effects of TLR4. TAK242 reversed the NALP3 activation and caspase-1 overexpression induced by soluble UA, suggesting the full dependence of NALP3 and caspase-1 activation on TLR4 following stimulation with soluble UA. These findings support our hypothesis that TLR4 mediates soluble UA uptake and initiates the downstream injury induced by soluble UA.

Nevertheless, unlike the complete blockade of the NALP3 inflammasome and caspase-1 by TAK242, TAK242 only partially inhibited the upregulation of IL-1β following incubation with soluble UA. It has been reported that apart from the classical cleavage pattern for IL-1β by caspase-1, IL-1β can also be cleaved from pro-IL-1β by protease (48) which has been detected in both the proximal and distal renal tubules in normal renal tissue, as well as in patients with tubular disorders (49). Alternatively, cathepsin D has also been suggested as another possible means of IL-1β processing, as shown in the acidosis driven damage-associated molecular patterns (DAMPs) in cultured primary mouse glial cells (50). It is possible that there are other pathways available for IL-1β processing during soluble UA-induced tubular injury and the urate transporter may play a role; however, this requires further investigation.

In conclusion, our present findings suggest that soluble UA induces the formation of the NALP3 inflammasome, caspase-1 activation, IL-1β expression and ICAM-1 synthesis in human primary renal PTECs through a TLR4-dependent pathway, leading to the activation of innate immunity and the induction of pro-inflammatory cytokine production in human primary renal PTECs, as illustrated in Fig. 5. However, further studies on soluble UA using animal models and renal biopsy samples from patients with hyperuricaemia are warranted to verify these findings in vivo.

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