

Original Article

Uric acid induces TLR4-dependent innate immune response but not HLA-DR and CD40 activation in renal proximal tubular epithelial cells

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Received October 28, 2015; Accepted December 25, 2015; Epub February 1, 2016; Published February 15, 2016

Abstract: Hyperuricemia correlates with the development and progression of renal diseases. We have previously revealed that uric acid (UA) initiates immune responses through toll like receptors (TLR)4 dependent Nod-like receptor protein (NLRP) 3 inflammasome formation and interleukin (IL)-1 β processing, as well as major human leukocyte antigen (HLA)-DR (surface receptor for major histocompatibility complex II) and CD40 over-expression in primary renal mesangial cell. In this study we aimed to verify whether UA also activates these immune responses in a renal tubular epithelial cell line HK-2 cells. Human proximal tubular cells HK-2 were incubated with UA and lipopolysaccharides (LPS). Gene and protein expression level of innate immune markers TLR2, TLR4, NLRP3 and IL-1 β , adaptive immune markers HLA-DR and CD40, as well as intercellular adhesion molecule-1 (ICAM-1) were detected by RT-PCR, ELISA and Western blot, respectively. UA, like LPS, significantly enhanced the expression of TLR4, NLRP3, IL-1 β and ICAM-1 but failed to increase TLR2 expression in HK-2 cells. Neither LPS nor UA could increase HLA-DR and CD40 expression in HK-2 cells. TLR4 inhibitor, TAK242, significantly blocked the UA-induced NLRP3 and IL-1 β expression. Our findings suggest that UA induces TLR4 dependent innate immune response but not HLA-DR and CD 40 expression in renal proximal tubular cells.

Keywords: IL-1 β , NLRP3, renal tubular epithelial cells, TLR4, uric acid

Introduction

Hyperuricemia has been closely correlated with the development and the progression of chronic kidney disease (CKD). Tubulointerstitial inflammation and injury are common presentations in hyperuricemia-induced chronic renal injury, with increased macrophage and T cell infiltration observed in the tubulointerstitium of the kidneys [1]. Accumulating evidences have demonstrated that uric acid (UA) crystal-induced inflammation is a paradigm of innate immunity [2]. Innate immunity-related molecules, including Toll like receptor4 (TLR4), TLR2, Nod-like receptor protein (NLRP)3, caspase-1 and IL-1 β are essential in the development of hyperuricemia-induced gouty inflammation [3, 4]. UA has also been recognized as one of the “danger signals” that alerts the immune system to injurious conditions and has been shown to induce TLRs activation in lung injury [5], osteoarthritis [6] and preeclampsia [7]. However, whether elevated UA could also induce immune injury in the kidney and whether renal cells par-

ticipate in hyperuricemia induced inflammation is incompletely understood.

We have previously examined the immune potency of UA in renal mesangial cells which share similar properties as antigen presenting cells (APCs) and we have found that UA activated TLR4 dependent innate and adaptive immune responses with up-regulation of innate immunity markers including TLR4, NLRP3, IL-1 β , as well as adaptive immunity markers HLA-DR [surface receptor for major histocompatibility complex (MHC) II] and CD40, suggesting an important role of both innate and adaptive immunity in UA-induced mesangial injury [8]. Renal proximal tubular cells also express TLR2/4 and its TLR2/4 dependent signaling was reported to be activated by certain stimuli such as advanced glycation end-products modified low density lipoprotein (AGE-LDL) [9]. Moreover, tubular expression of MHC II, the molecule specifically expressed on APCs, was enhanced during their interaction with T cells [10]. Not only in autoimmune renal diseases,

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tubular cells are also potential to be switched to non-professional APCs with over-expressed MHCII and co-stimulatory molecule under stimulation of certain inflammatory factors like IFN- γ and LPS [11, 12]. As T cells and macrophages infiltration was common in the interstitial area of the kidney in hyperuricemic rats [13], and culture media of tubular cells incubated with UA induced macrophage recruitment *in vitro* [1], it is postulated that UA could be a stimulus and facilitate the interaction of tubular cells with immune cells. We have previously examined the innate immune responses in primary renal tubular cells and found that the innate immune response was activated in a TLR4 dependent way [14]. However, whether UA could also lead to adaptive immune response in renal tubular cells is not yet examined and needs further investigation.

Therefore, to further understand the mechanism of UA induced tubular cells immune injury, we investigated the effect of UA on innate immunity manifested by TLR2/4, NLRP3, IL-1 β and ICAM-1 expressions in cultured renal epithelial cells and tubular HLA-DR and CD40 expression for adaptive immune responses.

Materials and methods

Materials

Human tubular cell line HK-2, an immortal human proximal tubules cell line, was purchased from ATCC (Manassas, VA, USA). UA and LPS were purchased from Sigma (St. Louis, MO, USA). TAK242 was purchased from Chembest Research Laboratories Limited (Shanghai, China). MTT assay kit was purchased from Amresco (OH, USA). Reagent for RT-PCR was purchased from TaKaRa (Kyoto, Japan). Enzyme immunoassay kit for detection of IL-1 β was purchased from Biovolue (Shanghai, China) and soluble intercellular adhesion molecule (sICAM)-1 kit was purchased from eBioscience (SD, USA). TLR2, TLR4, NLRP3, CD40 and HLA-DR (surface receptor for MHC II) antibodies were purchased from Abcam (Cambridge, UK). Anti-mouse and anti-rabbit secondary antibodies were purchased from sinobio (Shanghai, China).

Cell culture

HK-2 cells were cultured in HK2 Keratinocyte-SFM medium (Gibco) supplemented with 10% FBS, antibiotics (Penicillin-Streptomycin solu-

tion, Sigma). We incubated cells at 37°C in 5% CO₂ and 95% air. In all experiments, a “growth arrest” period of 24 h was conducted for cells in serum-free medium before any stimulation.

Uric acid preparation

We dissolved UA in 1 M NaOH at a concentration of 16 mg/mL as previously described [15]. The solution was filtered (22 μ m pore size) and was stored at 4°C until use. Crystals were not detectable by polarizing microscopy, nor observed during cell incubation.

Viability assay of tubular cells under UA co-culture

To test the cell viability under incubation with different concentrations of UA, we seeded growth-arrested HK-2 cells into 96-well plates (0.25 \times 10⁵ cells per well) and exposed them to UA (0, 50, 100, 150, 200, 400 and 800 μ g/ml) for 24, 48 and 72 h, respectively. The cytotoxic effect of these stimulations on HK-2 cells was then examined by an MTT kit. Briefly, 20 μ l MTT was added to each well and incubated the cells at 37°C in 5% CO₂ for 4 h. We stopped the reaction with 150 μ l DMSO and measured the absorbance at 570 nm by an ELISA reader. The percentage changes in absorbance compared with that of the medium control (defined as HK-2 cells incubated with plain culture medium) were expressed.

Total RNA extraction and reverse transcription (RT)-polymerase chain reaction (PCR) of TLR2/4 and ICAM-1

We incubated growth arrested HK-2 cells with 200 μ g/ml of UA or 100 μ g/ml LPS for 4 h. Total cellular RNA was extracted using NucleoSpin RNA II total RNA extraction kit. The quality of the extracted RNA was monitored by formaldehyde agarose gel electrophoresis. Four microliter of total RNA was reverse transcribed to cDNA with Moloney Murine Leukemia Virus reverse transcriptase in a 20 μ l reaction mixture containing 50 ng random hexamer, 0.5 mmol/L dNTPs, and 20U of RNase inhibitor. The cDNA was stored at -20°C until further use. Gene expressions of TLR2, TLR4 and ICAM-1 by HK-2 cells were examined by PCR using specific primers (**Table 1**). For semi-quantification, human glyceraldehyde 3-phosphatedehydrogenase (GAPDH) primers (**Table 1**) were applied in every reaction as an internal control. PCR

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Table 1.Primer sequences and size of PCR products

Genes	5' to 3' sequences of PCR primers	Size of Products	Accession number
<i>TLR2-f</i>	CAA GGG CAG CTC AGG ATC	271 bp	U88878
<i>TLR2-r</i>	GAA AGG GGC TTG AAC CAG GA		
<i>TLR4-f</i>	CAA GAA CCT GGA CCT GAG CT	603 bp	U88880
<i>TLR4-r</i>	ATT GCA CAG GCC CTC TAG AG		
<i>ICAM-1-f</i>	GAG ACC CCG TTG CCT AAA	399 bp	NM_000201
<i>ICAM-1-r</i>	CCG CAG GTC CAG TTC AGT		
<i>GAPDH-f</i>	ATG GGG AAG GTG AAG GTC G	107 bp	X01677
<i>GAPDH-r</i>	GGG GTC ATT GAT GGC AAC AAT A		

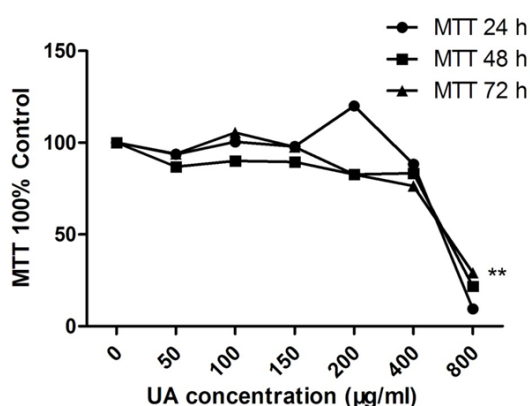


Figure 1. Viability of cultured HK-2 cells with incubation of UA. HK-2 cells were cultured with different concentrations of UA (0 to 800 µg/ml) for 24, 48 or 72 h. MTT assay showed that UA less than 400 µg/ml did not affect the cell viability at all the time points. Results are expressed as percentage changes in absorbance relative to that of the HK-2 cells incubated with plain medium alone. ** $P < 0.01$ versus plain medium. All results represent means \pm SD obtained from three independent experiments.

products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. Images of the gel were captured using the Gel Doc 1000 gel Documentation Densitometry System from Bio-Rad (Hercules, CA, USA). The product yield was expressed as a ratio to GAPDH.

ELISA of IL-1 β and sICAM-1 protein synthesis in cell culture supernatants

We incubated growth arrested HK-2 cells with 200 µg/ml of UA or 100 µg/ml of LPS (as positive control) for 48 h. Cells culture supernatants were collected and stored at -70°C until protein assay. Protein levels of IL-1 β and sICAM-1 were determined by commercial ELISA assay kits. The procedures were taken according to the manufacturer's instructions.

Western blot analysis of TLR2, TLR4, NLRP3, MHCII and CD40

After collecting the cell culture supernatant, the remaining cells were lysed with lysis buffer that contained protease inhibitor cocktails (Sigma, St Louis, MO, USA). Ten micrograms of total protein extracted from 10^6 cells were electrophoresed through a 12% SDS-PAGE gel before being transferred to a polyvinylidene difluoride (PVDF) membrane. After

blocking for 1 hour at room temperature in blocking buffer [5% bovine serum albumin in Tris-Buffered Saline (TBS) with 0.05% Tween-20 (TBST)], the membrane was incubated overnight with mouse anti-TLR2 (1:10000), mouse anti-TLR4 (3:500), mouse anti-CIAS1/NALP3 (1:1000), rabbit anti-HLA-DR (1:5000) and rabbit anti-CD40 (1:1000) and rabbit anti-GAPDH (1:10000) in TBST, respectively. The membrane was washed and incubated for 1 hour at room temperature with a peroxidase-labeled goat anti-rabbit or goat anti-mouse immunoglobulin. After further washing, the membrane was detected with ECL chemiluminescence (Amersham Pharmacia Biotech, Arlington, IL).

Statistical analysis

All data were expressed as means \pm 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. $P < 0.05$ was considered statistically significant.

Results

Viability of HK-2 cells and pH value of the medium under incubation with UA

The viability of HK-2 cells cultured with serial dilutions of UA for 24, 48 and 72 h was tested using MTT assay kit. The results showed that UA at the concentration of less than 400 µg/ml did not affect cell viability at all the time points (**Figure 1**, $P > 0.05$). We chose 200 µg/ml of UA for later experiments. This UA concentration is within the urine UA concentration range and mimics the UA concentration in the renal tubular cells. The pH values of the culture medium of HK-2 cells after the addition of UA were tested by pH measure (Merck, Darmstadt, Ger-

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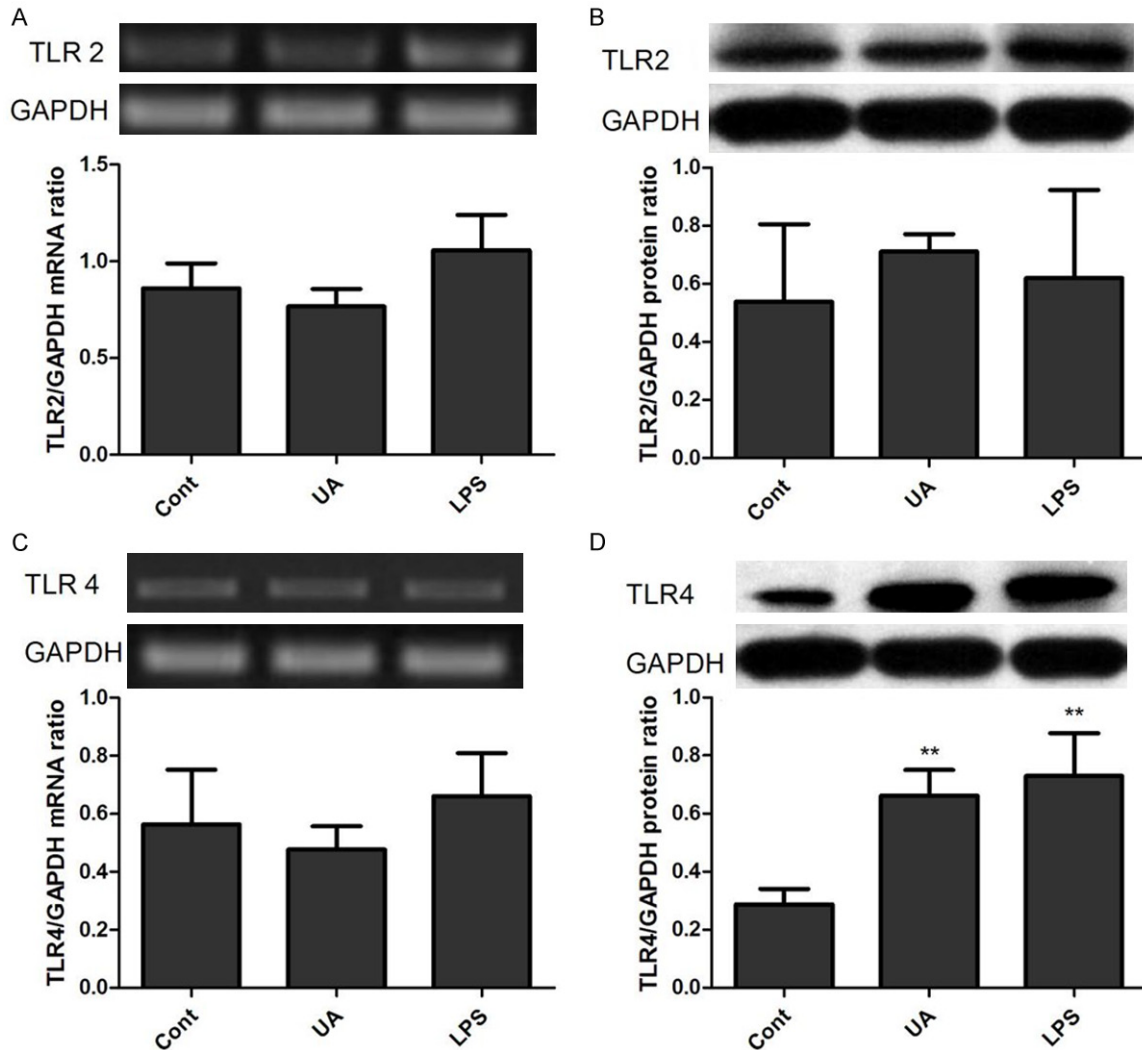


Figure 2. Gene and protein expressions of TLR2 and TLR4 in HK-2 cells cultured with UA. HK-2 cells were incubated with UA (200 $\mu\text{g}/\text{ml}$) or LPS (100 $\mu\text{g}/\text{ml}$, the positive control) for 4 h (gene expression) or for 48 h (protein expression). RT-PCR and Western blot results showed that only protein expression of TLR4 (D) but not its gene expression (C), was significantly increased in HK-2 cells cultured with UA or LPS. TLR2 expression was not upregulated by either UA or LPS (A, B). $**P < 0.01$ versus HK-2 cells cultured with plain medium. All results represent means \pm SD obtained from three independent experiments. Representative images of the corresponding PCR products and protein bands are shown at the top of each panel.

many). The pH values were all within the range from 7.1 to 7.4.

UA increased TLR4 but not TLR2 expression in HK-2 cells

To determine whether the innate immunity of HK-2 cells by UA was activated, TLR2 and TLR4, typical membrane receptors for innate immunity [2] were examined in HK-2 cells incubated with UA (200 $\mu\text{g}/\text{ml}$) for 4 h (gene analysis) or 48 h (protein analysis). LPS (100 $\mu\text{g}/\text{ml}$) was the positive control which can elicit TLR4 sig-

naling. Similar with LPS, UA only significantly increased TLR4 protein (Figure 2D, $P < 0.01$) but not its gene expression in HK-2 cells (Figure 2C). Neither LPS nor UA could increase the expression of TLR2 in HK-2 cells (Figure 2A, 2B).

UA enhanced NLRP3 expression in HK-2 cells

NLRP3 expression was measured by western blot after HK-2 cells incubated with 200 $\mu\text{g}/\text{ml}$ of UA for 48 h. LPS (100 $\mu\text{g}/\text{ml}$) was the positive control. As we showed in Figure 3, similar

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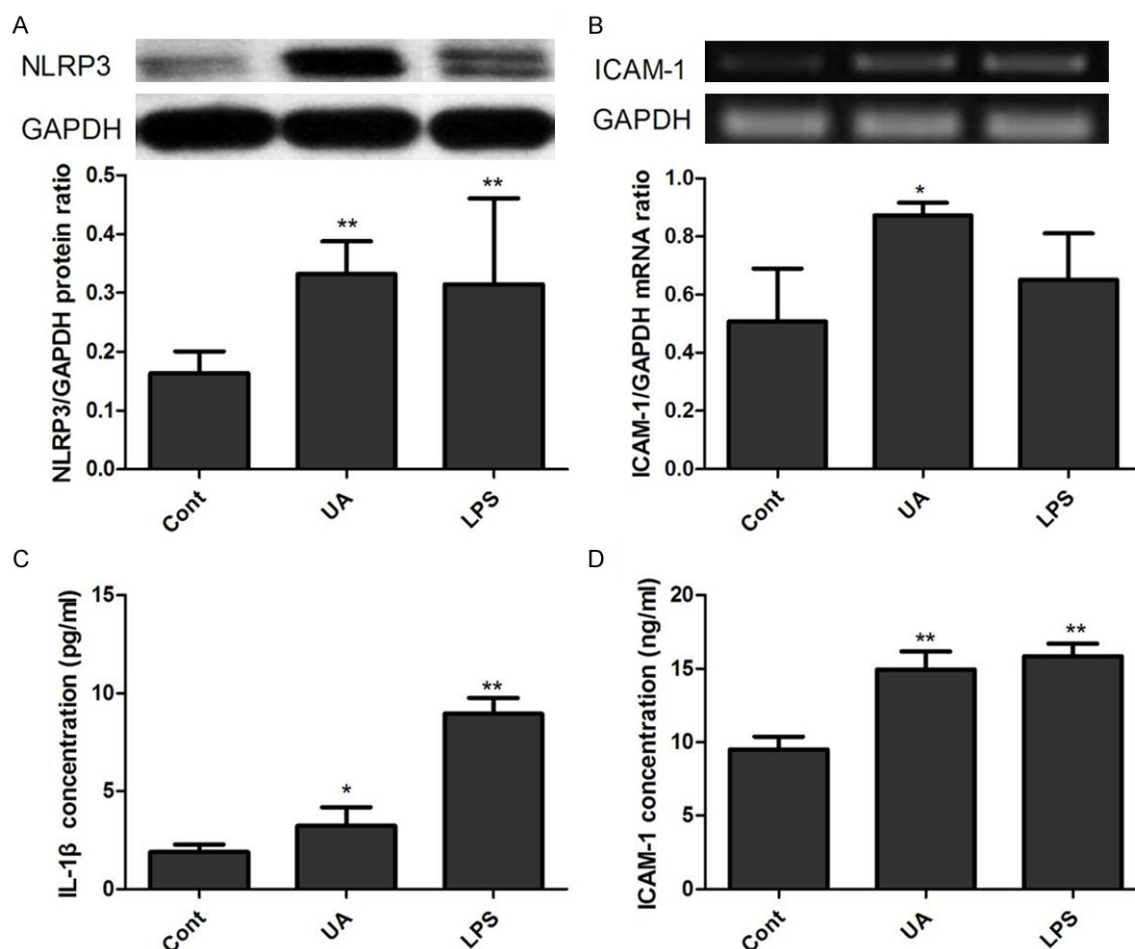


Figure 3. NLRP3, IL-1 β and ICAM-1 expressions in HK-2 cells cultured with UA. HK-2 cells were incubated with UA (200 μ g/ml) or LPS (100 μ g/ml, the positive control) for 48 h. NLRP3, IL-1 β and ICAM-1 protein synthesis were studied by Western blot and ELISA, respectively. Like LPS, UA significantly enhanced NLRP3 (A), IL-1 β (C) protein synthesis and ICAM-1 gene (B) and protein (D) expressions in HK-2 cells. * P <0.05, ** P <0.01 versus HK-2 cells cultured with plain medium. All results represent means \pm SD obtained from three independent experiments. Representative images of the corresponding protein bands are shown at the top of each panel.

with LPS, UA significantly enhanced NLRP3 protein expression (Figure 3A, P <0.01) in HK-2 cells.

UA increased IL-1 β protein synthesis in HK-2 cells

IL-1 β protein expression in HK-2 cells was examined by ELISA after stimulation of 200 μ g/ml UA for 48 h. LPS (100 μ g/ml) was the positive control which significantly enhanced IL-1 β production (Figure 3C, P <0.01). We noted that UA also significantly up-regulated IL-1 β protein expression (Figure 3C, P <0.05) in HK-2 cells.

UA increased ICAM-1 expression in HK-2 cells

The gene and protein expression of ICAM-1 in HK-2 cells were examined by RT-PCR and ELISA

after stimulation of 200 μ g/ml UA for 4 h and 48 h, respectively. LPS (100 μ g/ml) was the positive control which up-regulated protein expressions of ICAM-1 (P <0.01). The results showed that UA significantly up-regulated both ICAM-1 gene (Figure 3B, P <0.05) and protein expression (Figure 3D, P <0.01) in HK-2 cells.

UA failed to enhance MHCII or CD40 expressions in HK-2 cells

To determine whether tubular cells have been switched to non-professional APCs under UA stimulation, protein synthesis of HLA-DR (surface receptor for MHC II) and co-stimulatory molecule CD40 were examined by Western blot after stimulation of 200 μ g/ml UA or 100 μ g/ml LPS for 48 h. As shown in Figure 4 that neither

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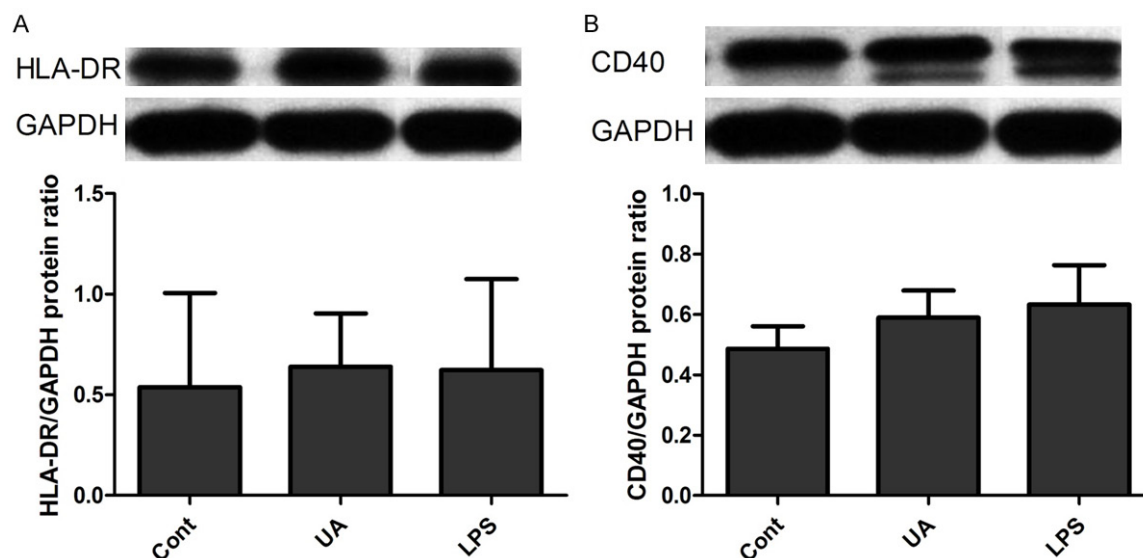


Figure 4. Protein expressions of HLA-DR and CD40 in HK-2 cells cultured with UA. HK-2 cells were incubated with UA (200 $\mu\text{g/ml}$) or LPS (100 $\mu\text{g/ml}$, the positive control) for 48 h to study the protein expressions of HLA-DR (surface receptor for MHC II) and CD40. Western blot results showed that neither LPS nor UA could increase HLA-DR (A) and CD40 (B) expressions in HK-2 cells. All results represent means \pm SD obtained from three independent experiments. Representative images of the corresponding protein bands are shown at the top of each panel.

LPS nor UA could enhance HLA-DR (**Figure 4A**) or CD40 (**Figure 4B**) protein expression.

TLR4-dependent NLRP3 expression and IL-1 β synthesis under UA stimulation

To determine whether the activation of TLR4 was specific for UA induced tubular immunity, TLR4 signaling specific inhibitor TAK242 (1 μM) was added to HK-2 cells 1 h before UA or LPS stimulation. Forty-eight hours later, the cell lysates were collected and NLRP3 and IL-1 β were examined by western blot and ELISA, respectively. TAK242 significantly blocked UA induced overexpression of NLRP3 (**Figure 5A**, $P < 0.01$) and IL-1 β protein synthesis in HK-2 cells (**Figure 5B**, $P < 0.05$).

Discussion

UA, one of the danger signals, leads to immune damages in various kinds of diseases, including gout. Here, we demonstrated that UA modulated renal tubular cells innate immunity with TLR4-dependent NLRP3 up-regulation as well as IL-1 β and ICAM-1 over-production. However, UA failed to induce HLA-DR and CD40 over-expression on tubular cells, suggesting the inability of UA to activate adaptive immune responses in renal tubular cells and UA was unable to

turn renal tubular cells into non-professional APCs.

Innate immunity has recently been found to be of great interests in several metabolic diseases [16]. It is closely associated with disease initiation and progression, for example in cardiovascular disease, the participation of immune response that contribute to inflammation has been reported [17]. It has been suggested that Th1 cells may affect the kidney, blood vessels remodeling directly via effects of the cytokines produced, or through their effects on perivascular fat, and hence contribute to increased blood pressure. The elevated blood pressure then generated neo-antigens which could act through damage-associated molecular pattern receptors or other mechanisms [17]. TLRs and NLRPs are typical mediators in the innate immune response. We have previously observed that UA activated TLR4 and NLRP3 in primary renal tubular [14] and mesangial cells [8] *in vitro*. Our current findings in renal tubular cell line HK-2 cells also showed that UA induced TLR4, NLRP3 and IL-1 β over-expression, demonstrating the activation of innate immune response by UA in renal tubular cell line, which suggested that renal tubular cell line HK-2 was also applicable for *in vitro* immune studies.

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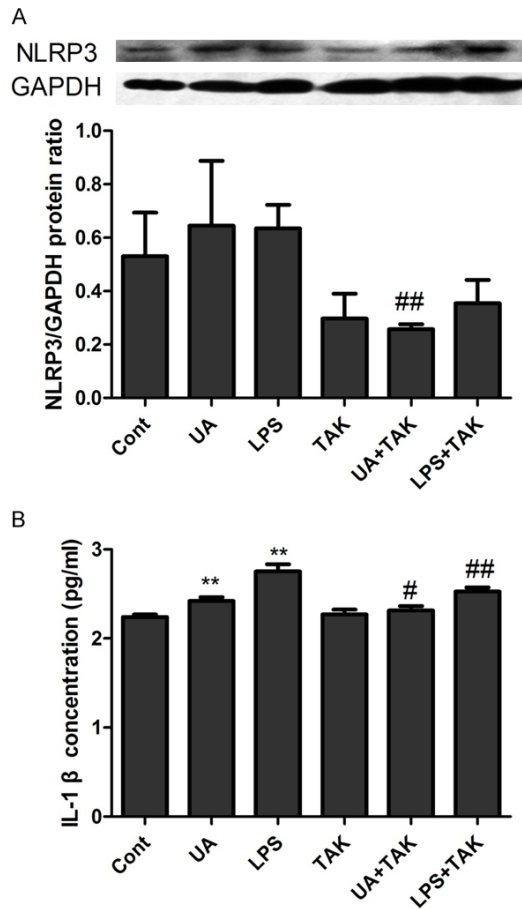


Figure 5. TLR4-dependent NLRP3 expression and IL-1 β protein synthesis under UA. To determine whether the activation of TLR4 was specific for UA induced tubular injury, TLR4 signaling specific inhibitor TAK242 (1 μ M) was added to HK-2 cells 1 hour before UA or LPS stimulation for 48 h. NLRP3 and IL-1 β protein synthesis were studied by Western blot and ELISA, respectively. TAK242 significantly blocked UA induced overexpression of NLRP3 (A) and IL-1 β protein synthesis (B) in HK-2 cells. ** $P < 0.01$ versus HK-2 cells cultured with plain medium, # $P < 0.05$, ## $P < 0.01$ versus HK-2 cultured with UA or LPS but without TAK242. All results represent means \pm SD obtained from three independent experiments. Representative images of the corresponding protein bands are shown at the top of each panel.

The process of UA into tubular cells has long been identified that UA has its specific transporters on both sides of tubular cells which mediate the absorption and the excretion of UA [18]. Many of the detrimental effects of UA are mediated through these transporters [1, 19]. However, urate transporter inhibitor could not completely block the pro-inflammatory effect of UA, suggesting the activation of other systems by UA may also account for the injury [20]. TLRs

are typical membrane receptors for the innate immunity and are among the first line defenses during innate immunity [21]. TLR2 and TLR4 are both recognized as receptors of UA and play significant roles in amplifying inflammatory effects [3]. Tubular cells also express these two receptors [22]. In the present study, we only noticed TLR4 but not TLR2 up-regulation in tubular cells under UA stimulation. TLR4 signaling inhibitor-TAK242 significantly blocked the downstream NLRP3 and IL-1 β synthesis induced by UA, indicating that these effects of UA are TLR4-dependent. The TLR4 dependent effect was also found in podocyte [23], mesangial cells [24] and experimental kidney transplantation [25]. Our results were in accordance with the findings by Lin M *et al.* in diabetic nephropathy that only TLR4, but not TLR2, mediated tubular damage [26]. In advanced glycation end-products modified low density lipoprotein (AGE-LDL) stimulated proximal tubular cells, both TLR2 and TLR4 were showed to interact with AGE-LDL, however, compared with TLR2 siRNA, TLR4 siRNA showed stronger inhibition on AGE-LDL-induced IL-6 and IL-8 production [9]. In endothelial cells, Mudaliar *et al.* also observed that fluctuating glucose concentrations maximally up-regulated TLR4 but not TLR2 expression and only TLR4 signaling inhibition suppressed the synthesis of proinflammatory chemokines and cytokines [27]. Therefore, our evidence shows that TLR4 plays a more important role in mediating immune responses in the UA lead immune injury in HK-2 cell model than TLR2, however, further examination is needed for validation.

In adaptive response, the components “adapt” to the changes and lead to immune responses from resting status. Renal epithelial cells are immune privileged for adaptive immune responses as they are surrounded by a dense network of immune cells, which provides an environment for the communication of tubular cells with immune cells. Actually, renal epithelial cells are similar in many functional and phenotypic characteristics with mononuclear phagocytes, such as secretion of chemokines in response to stimulation with TLR ligands and expression of MHC I and II, as well as co-stimulatory molecules [28]. All cell types can express MHC class II, but normally only professional APCs such as macrophages, B cells, and dendritic cells (DCs) express. Typical APC takes up an antigen and then performs antigen process-

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ing. MHC II expression has also been observed on renal tubular cells [29]. There was evidence suggesting that proximal tubules can also present antigen to T cells [30]. Stimulation like interferon (IFN)- γ [31], LPS [32] and transforming growth factor beta (TGF- β) [33] could differentially regulate class II MHC and co-stimulatory factor CD80 expression in renal tubular epithelial cells, indicating the potential antigen-presenting capability of renal tubular epithelial cells *in vitro*. HLA-DR, the membrane receptor of MHC II, has been shown to be linked to the severity of both tubular atrophy and tubulointerstitial fibrosis [34]. However, we failed to notice over-expression of HLA-DR in HK-2 cells after UA stimulation.

In addition, adaptive immunity also needs the activation of co-stimulatory molecules. Typically, there are mainly three co-stimulatory molecules for the process of antigen presentation, CD80, CD86 and CD40. Co-stimulatory factor CD40 is a membrane receptor which is expressed by a large variety of cell types including mesangial cells [35]. The CD40/CD40-L co-stimulation pathway also mediates immune injury in adriamycin nephrosis [36]. It is shown that only CD40 is stimulatory in tubular cells upon the co-incubation of IFN [37], hence we focused on CD40 expression in the current study.

Though we have observed the activation of adaptive immunity molecules in mesangial cells under UA stimulation [8], we failed to observe any HLA-DR or CD40 up-regulation in HK-2 cells. The UA induced HLA-DR and CD40 activation may be cell types-specific. In macrophage, UA crystal has been shown to increase macrophage MHC II and its co-stimulatory molecule over-expression and thus enhance the antigen presenting ability [38]. It is possible that macrophage and mesangial cells possess complete APC property and response with both the activation of innate and adaptive immunity to stimuli. However, renal tubular may not be a typical immune cell but only partially responds with only the activation of innate immunity. Another study also supported our findings and they found that renal tubular cells did not constitutively express significant amounts of co-stimulatory molecules. Compared to DCs, renal tubular cells only induced suboptimal T cell activation. The lack of co-stimulatory signals

between renal tubular epithelial cells and T cells greatly impaired the antigen-specific T cell activation [37], therefore it is possible that UA fails to induce antigen presenting response in tubular cells and could not trans-differentiate tubular cells to non-professional APCs.

Conclusion

Our findings suggest that UA induces NLRP3 over-expression and IL-1 β synthesis through a TLR4 dependent pathway in renal tubular cells. Neither LPS nor UA could increase HLA-DR and CD40 expression in renal tubular cells, which suggests that UA induces TLR4 dependent innate immune but not adaptive immune responses in renal proximal tubular cells.

Acknowledgements

The results in this paper were presented in the poster form in World Congress of Nephrology 2013 (May 31st to June 4th, Hong Kong). This manuscript has been seen and approved by all authors and that it is not under consideration for publication elsewhere in a similar form, in any language. This research is supported by National Science Foundation of China (Grant number 30900684/C140405), Shanghai Municipal Commission of Health and Family Planning (Grant number 20134075) and Shanghai medical guide of science and technology projects (Grant number 114119a6200).

Disclosure of conflict of interest

None.

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