Hyperuricemia-induced NLRP3 activation of macrophages contributes to the progression of diabetic nephropathy

Running title: NLRP3 mediates hyperuricemia-induced IL-1β in DM

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Abstract

Background: IL-1β-secreting NLRP3 inflammasomes play a pivotal role in triggering innate immune responses in metabolic disease. We investigated the role of soluble uric acid in NLRP3 inflammasome activation in macrophages to demonstrate the effect of systemic hyperuricemia on progressive kidney damage in type 2 diabetes.

Methods: THP-1 cells, human acute monocytic leukemia cells, were cultured to obtain macrophages, and HK-2 cells, human renal proximal tubule cells, were cultured and stimulated with uric acid. In vivo, we designed four groups as follows: 1) Long-Evans Tokushima Otsuka (LETO) rats; 2) Otsuka Long-Evans Tokushima Fatty (OLETF) rats; 3) OLETF + high-fructose diet (HFD) for 16 weeks; and 4) OLETF + HFD + allopurinol (10 mg/dL administered in the drinking water).

Results: Soluble uric acid stimulated NLRP3 inflammasomes to produce IL-1β in macrophages. Uric acid-induced MitoSOX mediates NLRP3 activation and IL-1β secretion. IL-1β from macrophages activates NF-κB in co-cultured proximal tubular cells. In vivo, intrarenal IL-1β expression and macrophage infiltration increased in HFD-fed OLETF rats. Lowering the serum uric acid level resulted in improving the albuminuria, tubular injury, macrophage infiltration, and renal IL-1β (60% of HFD-fed OLETF) independent of glycemic control. Direct activation of proximal tubular cells by uric acid resulted in CXCL12 and HMGB1 release and accelerated macrophage recruitment and the M1 phenotype.

Conclusions: Taken together, these data support direct roles of hyperuricemia in activating NLRP3 inflammasomes in macrophages, promoting chemokine signaling in the proximal tubule and contributing to the progression of diabetic nephropathy through cross-talk between macrophages and proximal tubular cells.
Key words: NLRP3 inflammasome, Uric acid, Macrophage, Diabetic nephropathy
**Introduction**

Hyperuricemia without gouty arthropathy is a common characteristic of type 2 diabetes, and this condition usually worsens as diabetic nephropathy progresses. In contrast to the outdated concept that hyperuricemia is simply a consequence of metabolic syndrome, recent clinical studies have demonstrated that hyperuricemia is an independent risk factor for chronic kidney disease in type 2 diabetes (2, 41). *In vitro* studies have reported that uric acid induces endothelial injury by releasing high mobility group box-1 (HMGB1) from endothelial cells, stimulating Toll-like receptor (TLR) pathways (29). Uric acid also activates vascular smooth muscle cell proliferation by producing chemotactic factors and by activating the renin-angiotensin system (11, 13). Previous studies have indicated that uric acid-induced injury is associated with sterile inflammation. This finding corresponds with our previous finding that reduced serum uric acid levels attenuate TGF-β1-induced profibrogenic injury of diabetic nephropathy in type 2 diabetes (16). Although large-scale epidemiologic and experimental studies have suggested that hyperuricemia is an independent risk factor in the development and progression of diabetic nephropathy, the action mechanism behind hyperuricemia and the progression of diabetic nephropathy remains to be clarified.

Type 2 diabetes is a chronic low-grade inflammatory disease. We reported that intrarenal interstitial CD3⁺ cell infiltration correlates with the degree of proteinuria in type 2 diabetic nephropathy patients (26). Genetic polymorphisms of IL-1, IL-1 receptor (IL-1R), and TNF-α were also significantly associated with an increased risk of end-stage renal disease in type 2 diabetes (19). These results indicate that immune cells and their related pro-inflammatory cytokines are associated with the progression of diabetic nephropathy. In particular, chronic low-grade inflammation in type 2 diabetes is associated with the activation of innate immune
systems (1, 12). Macrophage infiltration is accelerated by diabetic nephropathy related to metabolic stress, oxidative injury, chemokines, and adhesion molecules, resulting in increased pro-inflammatory cytokines (10, 35). However, it is unclear whether hyperuricemia-related macrophage activation initiates and amplifies inflammation in type 2 diabetic nephropathy.

Membrane TLRs and cytoplasmic nucleotide-binding oligomerization domain receptor (NOD)-like receptors (NLRs) (8) are the two major sensors of macrophages that are related to sterile inflammation. A recent interesting report demonstrated that compared with healthy controls, type 2 diabetes patients have significantly increased NLR protein (NLRP)3, apoptosis-associated speck-like protein containing a CARD (ASC), and proinflammatory cytokine protein expression in monocyte-derived macrophages (18). NLR family members sense danger signals, including pattern-associated molecular pattern (PAMP) and danger-associated molecular pattern (DAMP). The recognition of PAMP and DAMP by NLRP3 in the cytosol promotes inflammasome activation and IL-1β and IL-18 production. Although monosodium urate crystals and calcium pyrophosphate dehydrate crystals activate the NLRP3 inflammasome, which is considered to be a major pathogenic mechanism of gout and pseudogout (23), there is no report whether more prevalent hyperuricemia activates NLRP3. The present study hypothesized that chronic hyperuricemia-induced sterile inflammation is related to NLRP3 inflammasome activation in macrophages. This study aimed to clarify whether hyperuricemia-induced NLRP3 inflammasome activation contributes to the progression of diabetic nephropathy.
Methods

Cell culture
The human monocyte cell line THP-1 (ATCC, MD, USA) is part of the mononuclear phagocyte series and can be converted into activated macrophages with phorbol myristate acetate (PMA, Sigma Chemical Co., MO, USA). THP-1 cells were maintained in RPMI 1640 supplemented with 10% FBS and 0.05 mM 2-mercaptoethanol. Human kidney-2 (HK-2) cells (a human kidney proximal tubular epithelial cell line) were purchased from KCLB (the Korean cell line bank, Seoul, Korea). HK-2 cells were cultured in RPMI 1640 with 10% FBS and 1% P/S at 37°C in a humidified 5% CO2 incubator. Uric acid and allopurinol (Sigma Chemical Co., MO, USA) were added at different concentrations for various time periods. For the uric acid treatment, THP-1 and HK-2 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, and then, uric acid was added in different concentrations for various periods of time as scheduled. The cells were then collected at different time points for further characterization. Probenecid (Sigma Chemical Co., MO, USA) was used as a pharmacological inhibitor of uric acid.

Co-culture experiments
HK-2 cells were seeded on the lower side of Transwell growth supports (Corning, Germany), and PMA-treated THP-1 macrophages were added to the upper compartment. HK-2 and THP-1 cells were incubated for 12 hours in different concentrations of uric acid. Twelve hours later, the upper inserts that contained THP-1 cells were discarded. The HK-2 samples were then washed and assayed.
Chemotaxis analysis

The cell culture supernatants were added to the lower chambers of Transwell plates, and THP-1 cells with 7.5 nM PMA were added to the upper chambers. Cells were added to the uric acid-stimulated supernatant for 12 hours in HK-2 cells. Following cell incubation, cells on the top surface of the insert were removed by wiping with a cotton swab. Cells that migrated to the bottom surface of the insert were fixed in 4% paraformaldehyde, stained in DAPI, and then observed under a confocal microscope (Carl Zeiss LSM 700, Oberkochen, Germany). Next, the cells were stained by 0.2% crystal violet, and the absorbances of the test samples were measured at 570 nm.

Isolation of total RNA, RT, and real-time PCR

Total RNA was extracted from kidney tissue using the Total RNA Isolation Kit (MACHEREY-NAGEL, Germany). Real-time PCR was performed using SYBR Green PCR Master mix (FastStart Universal SYBR Green Master, Roche). The primer sequences used are shown in Table 1. Each sample was run in triplicate in separate tubes to permit quantification of the target gene expression normalized to GAPDH or 18s expression.

Western blot analysis

Cells and kidney cortical tissues were washed with PBS and lysed in ice-cold lysis buffer containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Proteins were separated with 10% PAGE and electroblotted onto a PVDF membrane (Millipore, Madrid, Spain). The membrane was incubated with primary antibody raised against NLRP3 (1:1,000, Abcam, Cambridge, UK), IL-1β (1:1,000, Cell Signaling Technology, MA, USA),
ASC, Caspase-1 (1:1,000, Santa Cruz, CA, USA), NF-κB (1:1,000, Cell Signaling Technology, MA, USA), or HMGB1 (1:1,000, Abcam, Cambridge, UK) and were subsequently stained with horseradish peroxidase-conjugated goat anti-rabbit or mouse immunoglobulin G (1:10,000, Santa Cruz, CA, USA). The immunoreactive bands were detected by chemiluminescence (ECL, BioFX Laboratories, Inc. MD, USA). β-actin (1:10,000, Santa Cruz, CA, USA) was used as an internal control.

**Enzyme-linked immunosorbent assay (ELISA)**

IL-1β was measured in rat kidney tissue by ELISA (Cayman Chemical, USA). The DNA binding activity of NF-κB was measured in co-cultured HK-2 cells by ELISA (Cayman Chemical, USA).

**Immunocytochemistry**

Cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% NP-40. After blocking, the slides were incubated with antibodies to NLRP3, ASC (1:100, Santa Cruz, CA, USA) and HMGB1 (1:100, Abcam, Cambridge, UK) overnight at 4°C. The cells were stained with secondary antibody conjugated with FITC or Texas Red and DAPI for nuclear staining and viewed under a confocal microscope.

**Measurement of mitochondrial superoxide generation**

Mitochondrial superoxide generation in live cells was assessed with MitoSOX Red (Molecular Probes, Eugene, USA). THP-1 cells were incubated with 5 μM MitoSOX Red at 37°C for 10 minutes and then examined using flow cytometry (BD FACSCalibur Flow Cytometry, USA) and a confocal microscope.
**Small interfering RNA (siRNA) knockdown experiments**

Duplex siRNAs targeting NLRP3 and a control siRNA were purchased from Bioneer Inc. (Seoul, Korea). The cells were transfected using 20 nM siRNA mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), after which the cells were utilized 24 to 72 hours later for functional studies. The knockdown efficiency was assessed by Western blot analysis using anti-NLRP3 and anti-β-actin antibodies.

**Animals and experimental design**

OLETF male rats and LETO male rats were supplied by the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan). The rats were divided into four groups of 10 rats each and were treated from 30 weeks to 46 weeks: (1) the non-diabetic control group (LETO) fed standard chow; (2) the diabetic group (OLETF) fed standard chow; (3) the high-fructose diet diabetic group (OLETF + HFD); and (4) the allopurinol-treated group fed a high-fructose diet (allopurinol was administered in the drinking water at a dose of 10 mg/dL) (OLETF + HFD + allopurinol). Intraperitoneal glucose tolerance tests (IPGTT) were performed at 46 weeks. The blood glucose levels were measured at 0, 30, 60, 90, and 120 minutes after 1 g of glucose/kg of body weight was injected intraperitoneally after 6 hours of fasting. The body weight, kidney weight, fasting blood glucose, glycosylated hemoglobin (HbA1c) (DCA 2000 system; Bayer Diagnostics, Elkhart, Ind., USA) and chemistry were measured at the time of sacrifice. Each rat was housed individually for 16 hours in metabolic cages for the collection of urine. Commercially available kits were used to measure urinary albumin (ALPCO, Salem, NH, USA) and urinary creatinine (R&D Systems, Minneapolis, MN). The systolic blood pressure was measured using tail-cuff plethysmography (ADInstruments,
All animal experiments were performed in compliance with the guidelines of the Animal Research Ethics Committee of Kyung Hee University, Seoul, Korea.

**Histology**

Pathologists used light microscopy (200 x magnification) with periodic acid-Schiff stain to evaluate the tubular injury in the tissue samples. The percentages of atrophic tubules (i.e., tubular dilation, detachment of tubular epithelial cells, and condensation of tubular nuclei) were assessed by examining at least 400 renal cortical tubules in randomly selected fields for each sample [1]. The kidney sections were incubated with primary antibodies for ED1 (CD68) (1:50, Serotec, Oxford, UK) using a biotin-free polymeric horseradish peroxidase (HRP)-linker antibody conjugate system in a Bond-max automatic slide stainer (Vision BioSystems, Victoria, Australia).

**Statistical analysis**

All values are expressed as the means ± SE. The results were analyzed using the Kruskal-Wallis nonparametric test for multiple comparisons. Significant differences in the Kruskal-Wallis test were confirmed by the Wilcoxon rank-sum and Mann-Whitney U-tests (used to compare mean differences); P-values < 0.05 were considered to be statistically significant.
Results

Uric acid induces NLRP3-mediated IL-1β secretion in macrophages

To confirm the direct effect of uric acid on macrophages, we evaluated the conformational change and IL-1β secretion of the NLRP3 inflammasomes using THP-1 cells. As shown in Fig. 1A, most NLRP3 and ASC were found in the cytosol under non-stimulatory conditions. Upon stimulation by uric acid, NLRP3 and ASC expression increased and co-localized (Fig. 1B). We also confirmed that uric acid increased NLRP3, ASC, and caspase-1 protein levels by immunoblotting (Fig. 1C-D). Following stimulation by uric acid, the activation of NLRP3 inflammasomes resulted in a marked increase of cleaved IL-1β in macrophages and macrophage supernatants (Fig. 1E-G). Probenecid was used for blocking organic anion transporter 1 to confirm NLRP3 activation and IL-1β secretion by extracellular soluble uric acid. With uric acid blocking by probenecid, NLRP3 and cleaved IL-1β did not increase in macrophages under uric acid stimulation (Fig 1H-I). To confirm whether NLRP3 modulated the IL-1β production under the uric acid stimulation in macrophages, THP-1 was transfected by 20 nM NLRP3 siRNA. Despite uric acid stimulation, NLRP3 siRNA-transfected THP-1 did not increase the NLRP3 or produce cleaved IL-1β (Fig. 1J-K). These results indicate that NLRP3 mediates extracellular soluble uric acid-induced IL-1β secretion in macrophages.

Uric acid activates NLRP3 inflammasomes through mitochondrial ROS in macrophages

We further investigated how uric acid activates NLRP3 inflammasomes in macrophages. As shown in Fig. 2A, THP-1 stimulated with uric acid produced greater mitochondrial ROS, which was relieved by pretreatment with the mitochondrial targeting antioxidant Mito-TEMPO or probenecid. MitoSOX-positive cell counts also showed that uric acid-stimulated
 mitochondria superoxide generation was decreased by Mito-TEMPO (Fig. 2B). The blockade of mitochondrial ROS by Mito-TEMPO resulted in decreased NLRP3 activation and cleaved IL-1β secretion in macrophages (Figs. 2C-F). These data suggest that mitochondrial ROS mediates uric acid-induced activation of NLRP3 inflammasomes in macrophages.

**IL-1β from macrophages induces NF-κB transcription in tubular cells**

Next, we investigated how IL-1β derived from macrophages affects tubular cells using co-cultures of THP-1 and HK-2 cells. Direct uric acid or LPS stimulation of HK-2 cells did not result in IL-1β secretion despite increasing NLRP3 expression, and there was no IL-1R1 or IL-1 receptor-associated kinase (IRAK4) response (Fig. 3A-D). However, IL-1R1 and IRAK4 mRNA in HK-2 significantly increased when uric acid-stimulated THP-1 was co-cultured. Nuclear expression of the p65 subunit and NF-κB DNA binding activity in HK-2 cells showed significant increases when co-cultured with uric acid-stimulated THP-1 cells (Figs. 3E-H). However, there was no NF-κB activation when uric acid was used to stimulate the HK-2 cells directly. These results suggest that IL-1β derived from uric acid-induced NLRP3 inflammasome activation in macrophages results in increased tubular NF-κB activation.

**Physical and biochemical characteristics of the study animals**

We performed *in vivo* experiments using an HFD-induced hyperuricemia model of type 2 diabetes. This study compared various biochemical parameters in four experimental groups of rats, as shown in Table 2. We also confirmed the characteristics of the LETO + allopurinol group. Notably, there was no difference between the LETO and LETO + allopurinol groups. Compared to the OLETF rats, the HFD-fed OLETF group had higher kidney-to-body weight
ratios, epididymal fat, serum triglycerides, uric acid levels, and urinary albumin excretion. Treating the HFD-fed OLETF group with allopurinol for 16 weeks resulted in decreased kidney-to-body weight ratios, serum uric acid levels, and urinary albumin excretion compared with those of the OLETF group, but there were no differences in the triglycerides, HbA1c, and intraperitoneal glucose tolerance test (Fig. 4A and B). As shown in Fig. 4C and D, treating the HFD-fed OLETF group with allopurinol attenuated diabetic tubular injury compared to the HFD-OLETF group. Allopurinol also improved hyperuricemia-related inflammation and fibrosis in diabetic nephropathy (Fig 4 E-H). These findings suggest that the decreased serum uric acid levels improved HFD-induced diabetic nephropathy in the OLETF group without any changes in glycemic control.

**Increased IL-1β expression in HFD-fed OLETF kidneys**

Macrophage infiltration of the kidney significantly increased in the HFD-fed OLETF group compared to the OLETF group (Fig. 5A-D). Lowering the serum uric acid levels decreased the HFD-induced macrophage infiltration in the diabetic kidney. Lowering the serum uric acid level attenuated the HFD-induced IL-1β expression in the kidney (Fig. 5E-G). These findings demonstrate that hyperuricemia promotes NLRP3-activated macrophage infiltration and increases the secretion of IL-1β in the diabetic kidney.

**Uric acid induces CXCL12 and HMGB1 in tubular cells and increases macrophage recruitment and M1 polarization**

To investigate the direct effect of uric acid on tubular cells, we confirmed the expression of chemokines under uric acid stimulation in HK-2 cells. HMGB1 is considered to induce macrophages to express pro-inflammatory cytokines. We confirmed uric acid-induced
HMGB1 expression and release in tubular cells and macrophage polarization. The protein expression of HMGB1 and its translocation from the nucleus to cytoplasm increased after uric acid stimulation in HK-2 cells (Fig. 6A-C). The mRNA expression of CXCL12 increased up to six hours after the uric acid stimulation (Fig. 6D). A migration assay was used to confirm that increased CXCL12 can recruit macrophages. The number of migrated THP-1 cells was significantly increased by adding the uric acid-stimulated HK-2 cell supernatant (Fig. 6E-F). When blocking uric acid with probenecid, CXCL12 and HMGB1 did not increase under uric acid stimulation. Moreover, the THP-1 cells induced the mRNA expression of the M1 marker iNOs by the uric acid-stimulated HK-2 cell supernatant (Fig. 6G). These results suggest that uric acid-stimulated tubular cells release CXCL12 and HMGB1, which recruit macrophages and induce M1 polarization.
In the present study, we demonstrated that soluble uric acid stimulates NLRP3 inflammasomes through mitochondrial ROS generation in macrophages. Decreasing the uric acid treatment significantly attenuated the infiltration of NLRP3 inflammasome-activated and IL-1β-secreting macrophages, resulting in diabetic nephropathy improvement independent of glycemic control.

We hypothesized that hyperuricemia acts as one of the endogenous DAMPs and contributes to the progression of inflammation in diabetic nephropathy. The causes of chronic low-grade inflammation in diabetic nephropathy are hyperglycemia, hyperinsulinemia, advanced glycation end-products, reactive oxygen species, and the rennin-angiotensin system. These diabetes-related components or the production of endogenous DAMPs during disease progression have been proposed to activate TLRs and NLRs and can be directly sensed. In the present study, we demonstrated that hyperuricemia acts similarly to DAMPs and can activate NLRP3 in macrophages, which contributes to the progression of diabetic nephropathy. Despite the allopurinol-related decrease of only the uric acid level in the HFD-fed OLETF rats, the kidney-to-body weight ratio, urine microalbumin-to-creatinine ratio, tubular injury score, and renal fibrosis were improved according to the reduced serum uric acid levels. With regard to the use of allopurinol as a xanthine oxidase inhibitor, some previous studies have reported that it has anti-oxidant and anti-inflammatory properties in addition to uric acid-lowering properties (14). We also tested the effect of allopurinol in OLETF rats directly, and there was no difference in the urine microalbumin-to-creatinine ratio between the OLETF rats and allopurinol-treated OLETF rats; there was also no significant uric acid level reduction (data not shown). The systolic blood pressure also did not
show significant changes following HFD or allopurinol treatment in OLETF rats. Based on these results, we can assume that allopurinol has little effect without an accompanying reduction of uric acid levels in OLETF rats. Therefore, the protective role of diabetic nephropathy of allopurinol in the present study is associated with lowering the serum uric acid level in type 2 diabetics.

The average dose of allopurinol required to induce a 30 - 40% reduction in serum uric acid is 300 mg/day, but a 600 mg/day dose was required to reduce the levels by up to 60% in a human study (7, 28). In the present study, the HFD-fed OLETF rats were administered approximately 3 mg/day of allopurinol. Considering that the body weight of OLETF rats is approximately 600 mg, this is similar to a dose of 300 mg/day in a 60-kg person, which resulted in a 30% reduction in the serum uric acid level. A recent small sample size of clinical study showed that compared with the conventional group, a reduction of almost 30% in the serum uric acid level by allopurinol in type 2 diabetic patients was effective for maintaining urinary albumin secretion and the glomerular filtration rate over a period of three years (20). The effective allopurinol dosage for the treatment of diabetic nephropathy is not clear; however, a randomized clinical trial using allopurinol in type 1 diabetes mellitus patients to protect against kidney function decline is currently being conducted (22). The results of this study will guide clinical allopurinol treatment in diabetic nephropathy.

In the present study, we demonstrated that uric acid activated NLRP3 inflammasomes and IL-1β secretion in macrophages through mitochondrial ROS production. Recent reports have indicated that mitochondrial dysfunction and oxidative stress is an important activator of inflammasomes (27, 30). These results imply that soluble uric acid could activate NLRP3 inflammasomes via mitochondrial ROS production; this finding is consistent with previous results that uric acid acts as a pro-oxidant inside cells and can induce the stimulation of
NAD(P)H oxidase, which leads to mitochondrial dysfunction (31). We confirmed the characteristics of the soluble uric acid media by polar light microscopy and cathepsin B expression after uric acid stimulation to exclude the possibility that NLRP3 was activated by monosodium urate crystal formation and not by soluble uric acid in the preliminary study. There was no evidence of crystal formation by polar light microscopy and cathepsin B activation. Moreover, probenecid blockade of an organic anion transporter did not increase the NLRP3 expression and IL-1β secretion. Thus, extracellular soluble uric acid can activate NLRP3 inflammasomes.

A recent point of investigation regarding inflammasomes is the source of NLRP3-induced IL-1β. Systemic mononuclear cells and tissue-specific inflammasomes may have different mechanisms of action. Bakker et al. reported that NLRP3 shows a tissue-specific role in which leukocyte-associated NLRP3 was associated with tubular apoptosis, whereas renal-associated NLRP3 impaired wound healing in a renal ischemia reperfusion animal model (4). Conversely, NLRP3 or caspase-1 knock-out mice did not develop glomerular diabetic injury, even with wild-type bone marrow transplantation (33). To clarify the different roles and effects of NLRP3 when systemic or activated only in the kidney, a conditional knock-out animal model will be needed. We confirmed that there was hyperuricemia-induced NLRP3 expression in the proximal tubular cells; however, there was no IL-1β secretion. This result is consistent with previous studies showing noncanonical effects of NLRP3 in the kidney (5, 21, 38). Note that inflammasome-independent NLRP3 exists; thus, IL-1β non-secreting NLRP3 plays a role in ischemic reperfusion injury and TGF-β1-induced epithelial mesenchymal transition in the kidney (34, 38). A limitation of this study is that we did not investigate the role of IL-1β non-secreting NLRP3 in the kidney. Another study, however, reported that a mouse proximal tubular cell line secretes IL-1β following albumin injury (40); the role of
tubular cell-specific NLRP3 remains to be clarified. Recent other reports have suggested that
NLRP3 is expressed in podocytes secreting IL-1β and that its expression promotes podocyte
injury under hyperhomocysteinemia (37, 39).

The inflammatory process driven by chemokine-induced inflammatory cell recruitment and
cytokine production is a cardinal pathologic mechanism in diabetic nephropathy. In particular,
macrophage infiltration plays a pathological role in intrarenal chemokine production, and the
inflammatory response is also implicated in the progression of diabetic nephropathy (10). In
this study, proximal cells produced CXCL12 under uric acid stimulation and resulted in
increased macrophage recruitment. CXCL12 is highly active under pathological conditions
involving inflammation, ischemia, and proangiogenic environments, such as in malignancy or
autoimmune disease. The podocytes produce CXCL12, which contributes to proteinuria and
glomerulosclerosis, and anti-CXCL12 antibody attenuated podocyte injury in a murine type 2
diabetes model (32). In an ischemic reperfusion kidney injury model, however, CXCR7, a
receptor of CXCL12, is related to survival by mediating recruited transendothelial homing of
human renal progenitor cells (24). This functional switch of CXCL12, which involves a
proinflammatory role in recruiting T cells and monocytes or a recovering role by stem cell
homing, is dependent on the pathologic condition, but it is not fully understood. In our study,
the CXCL12 in tubular cells appeared to play a pro-inflammatory role by increasing
macrophage recruitment in the diabetic kidney. Notably, the recruited macrophages
demonstrated an M1 phenotype, which may be related to HMGB1 expression and release
from tubular cells by uric acid stimulation. A recent report demonstrated that HMGB1
exacerbated renal tubulointerstitial fibrosis through facilitating the M1 macrophage
phenotype (36). Taken together, hyperuricemia not only activates NLRP3 inflammasomes in
macrophages but also directly stimulates chemokine secretion in the proximal tubular cells,
which results in macrophage recruitment and M1 polarization, facilitating cellular cross-talk between macrophages and tubular cells in the type 2 diabetic kidney (Figure 7).

Macrophages produce IL-1β and activate IL-1β signaling through IL-1R and IRAK4. These cascades resulted in NF-κB activation in proximal tubular cells in the diabetic kidney. NF-κB is a well-known transcription factor that is responsible for regulating the expression of genes that are involved in inflammatory pathways, such as proinflammatory cytokines, chemokines, and adhesion molecules (3, 6). Increasing evidence from experimental diabetic nephropathy models demonstrates that NF-κB is activated by and contributes to macrophage infiltration (9, 17, 25). A recent study reported that an NF-κB inhibitor provided a protective effect against target organ damage in db/db mice through improved metabolic alterations, as well as by inhibiting profibrotic and proinflammatory processes (15). We confirmed that uric-acid-induced NLRP3 activation in macrophages resulted in an increase in tubular NF-κB, which is associated with tubulointerstitial fibrosis and macrophage infiltration in the diabetic kidney.

In summary, we demonstrated that extracellular soluble uric acid aggravates diabetic kidney injury by activating NLRP3 inflammasomes in macrophages and by promoting chemokine secretion in proximal tubular cells. These different systemic and renal effects of uric acid resulted in the recruitment of IL-1β-secreting macrophages to the diabetic kidney. We conclude that hyperuricemia plays an independent role in the progression of diabetic nephropathy by macrophage and tubular cell cross-talk.
Acknowledgements

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References


**Titles and Legends**

Figure 1. NLRP3-mediated uric acid-induced IL-1β secretion in macrophages. A. Localization of NLRP3 (Red) and ASC (Green) in THP-1 macrophages by confocal microscopy. B. Quantitation of NLRP3 and ASC colocalization. C and D. The protein expression levels of NLRP3, ASC, and Caspase-1 at different uric acid concentrations, assessed by immunoblotting. E and F. Maturation of pro-IL-1β under uric acid stimulation in macrophages. G. Concentration of secreted IL-1β in medium assessed by ELISA. H and I. NLRP3 and IL-1β with uric acid blocking by probenecid. J and K. NLRP3 and IL-1β in NLRP3 siRNA-transfected THP-1. \( ^p < 0.05 \) vs. PMA 7.5 nM treatment of THP-1 cells, \( ^{†}p < 0.05 \) vs. PMA 7.5 nM treatment of THP-1 cells + uric acid.

Figure 2. Uric acid-induced mitochondrial superoxide generation mediates NLRP3 inflammasome activation in macrophages. A. Flow cytometric analysis for MitoSOX after uric acid stimulation with or without Mito-TEMPO and probenecid. B. Measurement of MitoSOX fluorescence intensity by confocal microscopy. C and D. NLRP3 activation after uric acid stimulation with or without Mito-TEMPO. E and F. The maturation of pro-IL-1β expression after uric acid stimulation with or without Mito-TEMPO. \( ^p < 0.05 \) vs. PMA 7.5 nM, \( ^{†}p < 0.05 \) vs. PMA 7.5 nM treatment of THP-1 cells + uric acid.

Figure 3. Release of IL-1β from macrophage-mediated IL-1R1 signaling pathways in tubular cells. A and B. The expression levels of NLRP3 in HK-2 cells at different uric acid concentrations and times, as assessed by immunoblotting. C and D. No expression of IL-1β in HK-2 cells was detected by immunoblotting. E. HK-2 and THP-1 cells co-incubated for 12
hours with uric acid and IL-1R1 and IRAK4 mRNA expression detected by real-time PCR in HK-2 cells. F and G. NF-κB activation detected by nuclear fraction immunoblotting in HK-2 cells co-cultured with THP-1 cells after uric acid stimulation. H. DNA binding activity of NF-κB by ELISA. T+H, HK-2 and THP-1 cell co-culture, OD, optical density, #p < 0.05 vs. PMA 7.5 nM, †p < 0.05 vs. HK-2 cells + uric acid.

Figure 4. The effect of lowering uric acid levels. A. Serum uric acid concentration. B. Intraperitoneal glucose tolerance test. C. Tubular damage visualized with PAS staining in a) LETO, b) OLETF, c) OLETf + HFD, and d) OLETf + HFD + Allopurinol. D. Quantitation of the tubulointerstitial injury. E-H. Analysis of TNF-α, TGF-β1, Fibronectin and Type I Collagen mRNA expression in renal tissue by real-time PCR. *p < 0.05 vs. LETO, #p < 0.05 vs. OLETF.

Figure 5. The effects of lowering uric acid levels on macrophages in diabetic kidney. A. CD 68 macrophage marker. B. Quantitation of CD 68 staining. C and D. Immunoblotting of CD 68 in the kidney. E and F. The maturation of IL-1β expression. G. IL-1β in kidney tissue protein lysates measured by ELISA. *p < 0.05 vs. LETO, #p < 0.05 vs. OLETF.

Figure 6. Uric acid induces CXCL12 and HMGB1 in tubular cells and increases macrophage recruitment and M1 polarization. A. HMGB1 expression by uric acid in HK-2 cells. B. HMGB1 release in HK-2 cells by confocal microscopy C. After uric acid stimulation, CXCL12 mRNA expression in HK-2 cells detected by real-time PCR. D. Transwell migration assay showing migration capability in THP-1 macrophage with media from uric acid-stimulated HK-2 cells detected by confocal microscopy after DAPI staining. E and F. Cells
stained with crystal violet and then analyzed for absorbance at 570 nm using a microplate reader. The mRNA expression of iNOS and arginase in THP-1 macrophages with media from uric acid-stimulated HK-2 cells.

*p < 0.05 vs. Control (HK-2 cells), #p < 0.05 vs. THP-1 PMA 7.5 nM.

Figure 7. Hyperuricemia plays an independent role in diabetic nephropathy progression by macrophage and tubular cell cross-talk.
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<tr>
<td>IL-R1</td>
<td>5'-GTG ATC TGC AAA TGA AAT TG-3'</td>
<td>5'-TGC TTA AATAT GCT TGTGCA T-3'</td>
</tr>
<tr>
<td>IRAK4</td>
<td>5'-CAA CAT ATG TGC GCT GCC TC-3'</td>
<td>5'-GAC TTG AGG AGT CAG GTG GC-3'</td>
</tr>
<tr>
<td>CXCL12</td>
<td>5'-TGA GCT ACA GAT GCC CAT GC-3'</td>
<td>5'-TAG GCT TTG CCC AGG TTG AC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-CAT TGT GGAAGG GCTCAT GA-3'</td>
<td>5'-TCT TCT GGG TGG CAG TGA TG-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-ACTCCAGAAAAGCAAGCAA-3'</td>
<td>5'-CGAGCAGGAATGAGAAGAGG-3'</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>5'-TACAGGGCTTTCGCTTCAGT-3'</td>
<td>5'-TTGGTTGATAGGGCAAGGAC -3'</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>5'-TTATGACGACGGAAGACCT-3'</td>
<td>5'-GCTGGATGGAAAGATTACTC-3'</td>
</tr>
<tr>
<td>Type I collagen</td>
<td>5'- TCACCTACAGCAGCCTTG -3'</td>
<td>5'-GGTCTGTTCCAGGTTTGTG -3</td>
</tr>
<tr>
<td>iNOS</td>
<td>5'-GGTGGAAGCCGGTTAAACAAAGG-3'</td>
<td>5'-TGCTTGGTGGCGAAGATGA-3'</td>
</tr>
<tr>
<td>Arginase1</td>
<td>5'-TTCTCAAAAGGACAGCCTCG-3'</td>
<td>5'-AGCTTCCATTGGCTTCCC-3'</td>
</tr>
<tr>
<td></td>
<td>LETO</td>
<td>OLETF</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>573 ± 5</td>
<td>612 ± 5*</td>
</tr>
<tr>
<td><strong>Kidney/body weight ratio (%)</strong></td>
<td>0.44 ± 0.01</td>
<td>0.52 ± 0.02*</td>
</tr>
<tr>
<td><strong>Epididymal fat (g)</strong></td>
<td>5.5 ± 0.2</td>
<td>6.2 ± 0.3*</td>
</tr>
<tr>
<td><strong>Cholesterol (mg/dL)</strong></td>
<td>94 ± 4</td>
<td>113 ± 16*</td>
</tr>
<tr>
<td><strong>Triglyceride (mg/dL)</strong></td>
<td>30 ± 10</td>
<td>156 ± 51*</td>
</tr>
<tr>
<td><strong>HbA1c (%)</strong></td>
<td>3.6 ± 0.1</td>
<td>8.2 ± 0.5*</td>
</tr>
<tr>
<td><strong>HbA1c (mmol/mol)</strong></td>
<td>16 ± 1</td>
<td>66 ± 3</td>
</tr>
<tr>
<td><strong>BUN (mg/dL)</strong></td>
<td>12.8 ± 0.8</td>
<td>14.2 ± 2.2</td>
</tr>
<tr>
<td><strong>Serum creatinine (mg/dL)</strong></td>
<td>0.54 ± 0.04</td>
<td>0.59 ± 0.22</td>
</tr>
<tr>
<td><strong>UACR (μg/mg)</strong></td>
<td>30 ± 6</td>
<td>306 ± 83*</td>
</tr>
<tr>
<td><strong>SBP (mmHg)</strong></td>
<td>94 ± 2</td>
<td>98 ± 5</td>
</tr>
</tbody>
</table>

LETO, Long-Evans-Tokushima-Otsuka, OLETF, Otsuka-Evans-Tokushima-Fatty
HFD, High Fructose Diet
BUN, Blood urea nitrogen
UACR, Urinary albumin-creatinine ratio
SBP, Systolic blood pressure
Values are the mean ± S.E.M. *p < 0.05 versus LETO, #p < 0.05 versus OLETF
Figure 1.
Figure 2.
Figure 4.
Figure 5.

A

LETO  OLETF  OLETF+HFD  OLETF+HFD+Allopurinol
(a)  (b)  (c)  (d)

B

CTH3H  cell count/mm²

LETO  OLETF  OLETF+HFD  OLETF+HFD+Allopurinol

C

CD 68  OLETF  OLETF+HFD  OLETF+HFD+Allopurinol

D

CD86  Ratio

LETO  OLETF  OLETF+HFD  OLETF+HFD+Allopurinol

E

Chymo  LE-18  OLETF  OLETF+HFD  OLETF+HFD+Allopurinol

F

Chymo  B,  A  Ratio

LETO  OLETF  OLETF+HFD  OLETF+HFD+Allopurinol

G

Tumor  6  5  Ratio

LETO  OLETF  OLETF+HFD  OLETF+HFD+Allopurinol
Figure 7.