Hyperuricemia causes kidney damage by promoting autophagy and NLRP3-mediated inflammation in rats with urate oxidase deficiency

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Summary statement

We established a CRISPR/Cas9-mediated UOX gene-KO Wistar rat model of hyperuricemia. Using this model we show that autophagy and NLRP3-related inflammation were involved in uric acid nephropathy.
Abstract

Epidemiological research has shown that elevated serum urate concentration is a risk factor for the development of kidney disease, but the mechanisms underlying this process have not been elucidated. To examine the role of urate in the kidney, we performed functional disruption of urate oxidase (UOX) using the CRISPR/Cas9 system in Wistar rats. In comparison to wild-type (WT) rats, serum urate levels spontaneously and persistently increased in UOX-KO rats without a significant decrease in survival rate. The architecture and function of the kidneys in UOX-KO rats were impaired. Injury to the kidney resulted in increased interstitial fibrosis, macrophage infiltration, expression of NLRP3 and IL-1β, and activation of multiple cell-signaling pathways associated with autophagy, including the AMPK, p38 MAPK, ERK, and JNK pathways. Inhibition of autophagy with 3-MA abrogated the development of kidney damage and attenuated renal fibrosis, macrophage infiltration, and expression of NLRP3 and IL-1β in injured kidneys. In conclusion, the UOX-KO rat is a great model to study hyperuricemia-related diseases. Hyperuricemia-induced autophagy and NLRP3 dependent inflammation are critically involved in the development of renal damage. The inhibition of autophagy and inflammation are potential therapeutic strategies for uric acid nephropathy.
Introduction

Hyperuricemia is a metabolic disease caused by abnormalities in purine metabolism, mainly due to the increased formation or reduced excretion of urate. Approximately two thirds of the urate produced in humans is excreted by the kidneys. Urate undergoes extensive filtration, reabsorption, and secretion in the renal proximal tubule (Jalal, 2016). An increasing number of experimental and epidemiological studies have suggested that uric acid is an independent risk factor for renal disease (Mallat et al., 2016; Obermayr et al., 2008). However, the mechanisms underlying uric acid nephropathy (UAN) remain incompletely understood.

Hyperuricemia may induce renal inflammation via crystal-dependent and crystal-independent pathways (Braga et al., 2020). It is generally accepted that hyperuricemia induces renal inflammation in a crystal-dependent manner. Monosodium urate (MSU) crystals deposited in the tubular lumen or interstitial space can be recognized and engulfed by renal resident or infiltrating macrophages (Liu et al., 2015; Zhou et al., 2012). Upon stimulation with MSU crystals, chemokines such as CXCL-12 are produced, which induce directional proinflammatory cytokines such as interleukin (IL)-1β, IL-18, and interferons through the Src/Pyk2/PI3K signaling pathway (Valimaki et al., 2013). Nod-like receptor pyrin domain-containing protein (NLRP) 3, an important member of the NLRPs, senses danger signals, including pathogen-associated molecular patterns and damage-associated molecular patterns (DAMPs), in the cytosol and activates sterile inflammation (Chen and Chen, 2018;
Kim et al., 2015). Accordingly, the NLRP3 inflammasome was shown to be upregulated in both UAN patients and a rat model of UAN (Hu et al., 2019; Hu et al., 2018).

Autophagy is a crucial and rudimentary biological process involved in both physiological and pathological conditions (Liu et al., 2016). Physiologically, it is the degrading process of proteins and organelles mediated by lysosomes and participates in the regulation of cell metabolism and survival (Liu et al., 2016). Iconic proteins in autophagic membrane mainly include microtubule-associated protein 1 light chain 3 (LC3), Beclin-1, autophagy-related gene (Atg) 7 (Atg7), Atg12 and autophagy-adjusted protein (Havasi and Dong, 2016). Many studies have shown that a number of signal transduction pathways are involved in the regulation of autophagy, such as PI3K/AKT, MEK-ERK, and the signal from the cellular energy sensor AMP-activated protein kinase (AMPK), which controls the activity of mTOR as a upstream signal (Wang and Zhang, 2019). Recent studies have confirmed that autophagy also plays a functional role in hyperuricemia-induced inflammation (Maejima et al., 2013; Saitoh et al., 2008). Activation of autophagy may limit inflammasome activity induced by hyperuricemia through targeting ubiquitinated inflammasomes for degradation (Shi et al., 2012) and decreasing the production of reactive oxygen species (Isaka et al., 2016) and downstream inflammatory responses (Choe et al., 2014). This may be why gouty arthritis is self-limiting. In UAN, however, there have been contradictory reports regarding autophagy. One study
showed that persistent inhibition of autophagy promoted renal damage in rats with UAN fed a mixture of adenine (0.1 g/kg) and potassium oxonate (1.5 g/kg) daily for 3 weeks (Bao et al., 2018), whereas another study showed that upregulation of autophagy by Weicao exerted an anti-inflammatory and renal protective effect in UAN rats (Hu et al., 2018). These discrepancies may have been due to the differences in modeling schemes between the studies. Therefore, a suitable model for confirming the roles of autophagy and related inflammation in UAN is necessary.

In most mammals, serum urate concentrations are low (1–3 mg/dL [60–180 μM]) due to the enzyme urate oxidase (UOX), which degrades uric acid to 5-hydroxyisourate and allantoin. Humans and apes lack uricase due to inactivating mutations that had occurred during hominoid evolution, resulting in higher circulating urate concentrations. At present, drug induction using purine synthesis promoters and/or uricase inhibitors remain the primary strategies for establishing hyperuricemia models. However, serum urate levels are often unstable and fluctuate widely in animal models of drug-induced hyperuricemia. Alternatively, establishing a hyperuricemia model using urate oxidase gene knockout (KO) could lead to significant hyperuricemia, but few of these animals survive to maturity (Zhu et al., 2017). Encouragingly, Lu et al. (Lu et al., 2018) generated a UOX-KO mouse with a pure C57BL/6J genetic background using transcription activator-like effector nuclease (TALEN) technology. The laboratory rat, Rattus norvegicus, has been used as an animal model for human diseases for more than 150 years. Its size along with its
cognitive and physiological characteristics make the rat a useful model for cardiovascular disease, neurological disorders, and metabolic disorders. Rat models are superior to mouse models for testing pharmacodynamics, pharmacokinetics, and toxicity, partly because many of their detoxifying enzymes are very similar to those in humans (Alcantar and Rairdan, 2019). Therefore, there is a need for rat models that are not only easy to establish but also more representative of human pathologies. Clustered regularly interspaced short palindromic repeats (CRISPR) – CRISPR-associated protein (Cas) have provided a much simpler and more economic method for gene-targeted modification. This engineered nuclease generate a DNA double-strand break (DSB) at the targeted genome locus. The break activates repair through error-prone nonhomologous end joining (NHEJ) or homology-directed repair (HDR). In the absence of a template, NHEJ is activated, resulting in insertions and/or deletions (indels) that disrupt the target loci. In the presence of a donor template with homology to the targeted locus, the HDR pathway operates, allowing for precise mutations to be made (Ma et al., 2014). CRISPR/Cas9 is a simple and efficient tool to generate precise genetic modifications in rats, which will promote the accumulation of rat genetic resources and enable more precise studies of gene function (Alcantar and Rairdan, 2019).

In this study, a novel hyperuricemia model was established using Wistar rats via UOX gene KO using the CRISPR/Cas9 system. Using this model, we demonstrated that hyperuricemia induced renal damage with tubular injury and kidney fibrosis
through the activation of autophagy and NLRP3 inflammasome-mediated inflammation.

Results

Generation of UOX-KO rats with hyperuricemia

The KO scheme is shown in Figure 1A. The UOX gene consists of nine exons. We designed a 20-nucleotide targeting guide RNA (gRNA) construct (gRNA_{TRGT}) complementary to a region in exon 3 immediately upstream of the protospacer adjacent motif (PAM) unique to the UOX locus to discriminate alleles during Cas9 cleavage. Genomic DNA was extracted from pups for Sanger sequencing. Multiple peaks from heterozygous (HE) samples indicated the presence of novel genomic sequences originating downstream of the disparate PAM of UOX, whereas homozygous (HO) and WT rats produced single peaks (Figure 1B). Further alignment with normal WT rat sequences indicated that HO rats carried a 5-bp deletion in the UOX gene (Figure 1C). The expression of uricase in the liver was significantly lower in HO than in WT rats (Figure 1D).

HO rats had significantly higher serum urate levels than HE and WT rats at 8 weeks after birth. There were no differences in serum urate levels between male and female rats (Figure 1E). The observation period was extended from 4 to 52 weeks of age, and we found no differences in body weight between WT and HO rats of the same sex (Figure 1F). However, serum urate levels were consistently significantly higher in HO rats than in WT rats (Figure 1G). From 12 to 52 weeks of age, serum
urate levels in WT rats stabilized at approximately 100 μmol/L, whereas those in HO rats gradually increased from 280 to 400 μmol/L. To determine the survival rate of HO rats, we conducted Kaplan–Meier analysis over a 52-week observation period. The results showed that > 95% of the HO rats survived for > 1 year (Figure 1H). Genotyping at 1–2 weeks after birth revealed that the distribution of WT, HE, and HO rats was consistent with Mendelian inheritance, i.e., 28.57% (72 of 252), 47.62% (120 of 252), and 23.81% (60 of 252), respectively. Results of mating between homozygous UOX-KO rats showed that the number of births was normal, approximately 8–14 pups per dam, suggesting that the loss of the UOX gene did not lead to embryonic or neonatal death.

Taken together, these results indicate that the CRISPR/Cas9 system successfully constructed UOX-KO rats that exhibited persistent spontaneous hyperuricemia. The transgenic UOX-KO rat was named Wistar-Uox<sup>em2Cd5</sup>/IDM. Here, HO rats are referred to as UOX-KO rats.

**Renal function was impaired in UOX-KO rats**

Renal function was determined at 8 weeks of age in WT and UOX-KO rats of both sexes. Compared with the WT controls, serum creatinine and blood urea nitrogen (BUN) levels were significantly elevated in both male and female UOX-KO rats (Figure 2A and B). Compared with WT controls of the same sex, the 24-h urine volume of male UOX-KO rats increased 7.02-fold (4.01 ± 0.87 ml vs. 28.17 ± 7.7 ml, respectively), whereas that of female UOX-KO rats increased 4.05-fold (4.38 ± 2.04
ml vs. 17.72 ± 4.46 ml, respectively) (Figure 3C). Compared with the WT controls, the 24-h total urinary protein level also increased significantly in both male (6.15 ± 1.99 mg/24 h vs. 17.78 ± 3.51 mg/24 h, respectively) and female (4.92 ± 1.76 mg/24 h vs. 16.65 ± 3.51 mg/24 h, respectively) UOX-KO rats (Figure 2D). Compared with WT rats, urinary uric-acid excretion increased significantly in UOX-KO rats (Figure 2E), whereas total urine creatinine levels did not differ between groups (Figure 2F). These data indicate that uric acid can cause severe renal insufficiency, especially renal concentration dysfunction.

**Renal histopathology was impaired in UOX-KO rats**

Kidney morphology was examined at 4–52 weeks of age. Compared with male WT rats, male UOX-KO rats exhibited significant impairment in the kidneys characterized by a less smooth surface and structural damage (Figure 3A). Renal swelling and polycystic changes in UOX-KO rats occurred at approximately 8 weeks of age, followed by crystal deposition in the renal medulla and visible nephrolithiasis from 12 to 52 weeks of age. Renal histopathology was examined at 16 weeks of age. Urate crystals were detected in the kidney interstices in UOX-KO rats under polarized light (Figure 3B). Transmission electron microscopy revealed renal structural destruction, extensive fusion of podocytes, and thickening of the basement membrane in the kidneys of UOX-KO rats (Figure 3C). Hematoxylin and eosin staining of kidney tissues revealed the presence of atrophic glomeruli and tubular ectasia in UOX-KO rats (Figure 3D). Fibrosis is a pathological feature of UAN. Specifically,
Masson’s trichrome, collagen I, and alpha-smooth muscle actin (α-SMA) staining revealed that both glomerular and renal tubule fibrosis, especially tubulointerstitial fibrosis, were prominent in *UOX*-KO rats compared to WT rats (Figure 3E–G). Chronic inflammation was also observed in *UOX*-KO rats, manifesting as substantial macrophage (F4/80) infiltration and IL-1β expression, especially in the renal interstitium (Figure 3H and I). Taken together, these data indicate that hyperuricemia contributed to histopathological damage, mainly in the renal interstitium.

**Hyperuricemia increased autophagy in the kidney**

A number of studies have revealed the roles of autophagy in kidney diseases. To validate the role of autophagy in UAN, we further examined the appearance of autophagosomes and related autophagic vacuoles using electron microscopy. Autophagic vacuoles were rarely observed in the kidneys of WT rats, whereas numerous autophagic vacuoles were seen in the proximal tubular cells of *UOX*-KO rats (Figure 4A). Autophagy levels were also assessed by determining the LC3II/I ratio and levels of Beclin-1, p62, and Atg expression. In *UOX*-KO rats with hyperuricemic damage, the expression of Beclin-1 and Atg3 and the LC3II/I ratio increased, whereas p62 expression in the kidney decreased (Figure 4B). Autophagy can be regulated by different signaling pathways, such as the AMPK and MAPK/ERK pathways, which inhibit mTOR and then activate autophagy. Therefore, we examined these pathways and found that levels of p-JNK/JNK, p-ERK/ERK, and p-AMPK/AMPK expression increased, whereas the expression of p-mTOR/mTOR
decreased in the kidneys of UOX-KO rats (Figure 4C). These observations indicate that hyperuricemia can induce the formation of autophagic vacuoles, which are related to the regulation of AMPK- and MAPK-related pathways via uric acid.

**Inhibition of autophagy prevented renal dysfunction and alleviated renal histopathological changes in UOX-KO rats**

To further assess the roles of autophagy in UAN, we investigated the effects of 3-MA, a PI3K inhibitor, on renal function and fibrosis in UOX-KO rats. There were no significant changes in body weight or serum urate level in UOX-KO rats after 4 weeks of daily feeding with 3-MA (Figure 5A and B). However, serum creatinine and BUN levels decreased significantly in rats fed 3-MA (Figure 5C and D). The 24-h urine volume in the 3-MA group did not differ from that in the control UOX-KO group (Figure 5E), whereas the 24-h urinary protein and uric acid levels decreased significantly (Figure 5F and G). In addition, 24-h urine levels were not markedly altered by 3-MA.

Histopathological changes in the kidney were examined using immunohistochemistry (Figure 5I). Semiquantitative analysis of Masson’s trichrome-positive areas revealed that there was an approximately 5.3-fold increase in the deposition of extracellular matrix (ECM) components in hyperuricemic injured kidneys compared with control kidneys, whereas 3-MA treatment reduced ECM-component deposition by 56%. These results were also confirmed via collagen I and α-SMA staining. Kidney fibrosis is aggravated by chronic inflammation, which is
characterized by macrophage infiltration. As shown in Figure 5I, the number of F4/80-positive macrophages in the injured kidney increased in UOX-KO rats compared to WT rats, and 3-MA treatment markedly inhibited the infiltration of these cells. Levels of NLRP3 and IL-1β expression were also significantly reduced by 3-MA treatment. Hyperuricemic damage activated autophagy, characterized by an increased LC3II/I ratio and decreased expression of p62, and the AMPK, ERK, and JNK pathways were also activated. As shown in Figure 5J, 3-MA inhibited the autophagy process, and Atg3, Atg7, Beclin-1, and LC3 were significantly downregulated in 3-MA-treated kidneys. In addition, AMPK, ERK, and JNK activation was also suppressed by 3-MA. Furthermore, 3-MA inhibited the expression of NLRP3, cleaved caspase-1 and cleaved IL-1β (Figure 5K), suggesting that inhibiting autophagy improves renal function by reducing NLRP3-mediated inflammation in UAN.

**Discussion**

Hyperuricemia is critically associated with chronic kidney injury and the prevalence of UAN has increased worldwide. Here, we described a CRISPR/Cas9-mediated UOX gene-KO Wistar rat model of UAN. UOX gene KO in rats resulted in spontaneous sustained high serum uric acid levels and severe nephropathy, characterized by increased BUN and creatinine levels, increased urine volume and protein levels, renal fibrosis, and inflammatory cell infiltration in the kidney. Moreover, we found that autophagy was required for renal tubular injury and
the activation of multiple signaling pathways associated with renal fibrogenesis and inflammation. Using the autophagy inhibitor, 3-MA, we demonstrated that the inhibition of autophagy can preserve kidney function, reduce NLRP3-induced inflammatory responses, and suppress renal interstitial fibrosis in UAN.

Serum uric acid levels were higher in UOX-KO rats from as early as 2 weeks after birth, and this increase lasted for > 1 year compared with WT rats. Lu et al. reported that UOX-KO C57BL/6J mice had stably elevated serum urate levels of 420–520 μmol/L, which were 2–3-fold higher than those in WT mice (Lu et al., 2018)[19]. In other studies using UOX-KO mice, serum urate levels varied between 200 and 600 μmol/L (Hosoyamada et al., 2016; Inazawa et al., 2016)[21,22]. Compared with these UOX-KO transgenic mouse models, we observed a mild increase in serum urate level in our UOX-KO rat model. This may be because the average serum urate level of WT Wistar rats is lower than that of WT C57BL/6J mice (80 μmol/L vs. 200 μmol/L, respectively). Furthermore, our UOX-KO rats exhibited a high survival rate, with a 1-year survival of > 95%, which was significantly higher than in UOX-KO mouse models. These observations indicate that our UOX-KO Wistar rat model, which produced spontaneous 2–3-fold increases in serum urate levels that were maintained over a long period, can be a useful model for the long-term study of hyperuricemia and its complications.
The most prominent phenotype in the \textit{UOX-KO} rats was kidney damage. The \textit{UOX-KO} rats had severe UAN. First, serum biochemical indicators of renal function showed that serum creatinine and BUN levels were elevated in \textit{UOX-KO} rats. Second, urine tests indicated that urine volume and protein levels increased in \textit{UOX-KO} rats. Third, morphological examination of \textit{UOX-KO} rats revealed polycystic changes in the kidney at 8 weeks of age and systolic kidney-like changes at 52 weeks of age in combination with renal stone formation. Fourth, light microscopy revealed the occurrence of tubular dilatation, renal cortex atrophy, glomerular hypertrophy, glomerulosclerosis to some extent, and significant tubular fibrosis, and the deposition of urate crystals in this model. Fifth, macrophage infiltration, increased expression of inflammatory factors, and cell apoptosis were seen in the kidney, especially in the renal tubules, of \textit{UOX-KO} rats. The physiological characteristics of uric acid excretion may be responsible for hyperuricemia leading to kidney injury, especially tubular damage. Kidney damage occurred at a very early stage in the \textit{UOX-KO} model; thus, hyperuricemia was likely a direct cause of kidney damage.

The mechanism underlying UAN remains unclear. Autophagic responses to kidney damage can be beneficial or harmful depending on the pathological setting (Kimura et al., 2017). With regard to obstructed kidneys, Livingston et al. (Livingston et al., 2016) showed that the persistent activation of autophagy in kidney proximal tubules potentiates renal interstitial fibrosis by promoting the overexpression of
transforming growth factor beta, tubular cell death, and interstitial inflammation in a mouse model. However, in a rat model of unilateral ureteral obstruction, Yang et al. (Yang et al., 2020) suggested that autophagy may limit fibrosis by abolishing apoptosis. In this study, we demonstrated that chronic uric acid insult led to the induction of autophagy, which was represented by increased LC3II/I ratio and Beclin-1 expression and decreased expression of p62 in the kidney. Furthermore, AMPK, ERK, and JNK were activated and mTOR was inhibited in the kidneys of UOX-KO rats. Bao et al. (Bao et al., 2018) reported that the inhibition of autophagy by 3-MA attenuated hyperuricemic nephropathy in an adenine and potassium oxonate-induced hyperuricemia rat model. Consistent with previous research (Bao et al., 2018; Livingston et al., 2016), we found that 3-MA improved renal function, decreased urine microalbumin, attenuated pathological changes, inhibited activation of renal interstitial fibroblasts, and decreased the accumulation of ECM proteins in the hyperuricemic kidney. These observations imply that autophagy plays an important role in promoting hyperuricemic nephropathy.

MSU acts as a “danger signal,” designated as a DAMP, that warns the innate immune system of cellular insult, activates the NLRP3 inflammasome, and produces a proinflammatory response to repair damaged tissues (Martinon et al., 2006). In this study, we demonstrated that hyperuricemia could cause intrarenal crystal formation in the kidney, macrophage infiltration, and upregulation of NLRP3 and IL-1β expression. Autophagy inhibition may protect against hyperuricemic nephropathy by
inhibiting proinflammatory responses. Uric acid stimulates the activation of the NLRP3 inflammasome and PI3K/AKT signaling pathway in kidney epithelial cells (Lu et al., 2019) and the intestine (Chen et al., 2018). Tavares et al. (Tavares et al., 2019) reported that, in the absence of PI3Kγ activity, cleaved caspase-1 and IL-1β production in synovial tissue decreased after the injection of MSU crystals. In this study, we found that inhibition of autophagy by the PI3K inhibitor 3-MA downregulated the AMPK, ERK, and JNK signaling pathways, resulting in a decrease in NLRP3 activity and subsequent suppression of IL-1β release. These results suggest that autophagy is crucial for UAN, and it is necessary to regulate NLRP3 activation and IL-1β production. Drugs that regulate autophagy may contribute to the alleviation of renal injury and improvement in renal function.

In summary, we generated a CRISPR/Cas9-based UOX-KO Wistar rat model that exhibited persistent and stable hyperuricemia. This UOX-KO rat model would be an effective and reliable tool for studies of the mechanisms underlying hyperuricemia-related diseases and their potential treatments. Moreover, UOX-KO rats exhibited significant kidney damage, with autophagy activated in the kidney, whereas inhibition of autophagy by 3-MA alleviated UAN by reducing inflammation. Besides the traditional therapy used for UAN, regulation of autophagy or management of urate-induced NLRP3 inflammasomes may be novel effective therapeutic strategies.
Materials and methods

Generation of UOX-KO Rat

A specific scheme for gene editing strategy for rat UOX using a CRISPR-Cas9 system was shown in Figure 1A. Guide RNAs (gRNA) was designed targeting exons 3 in the UOX gene based on software tools predicting unique target sites throughout the rat genome (http://crispr.mit.edu/). gRNA sequence was 5’-CTCGCAGATGTTCCATAGCGA-3’ and PAM was AGG. gRNA were cloned into px330 vectors [pX330-U6-Chimeric_BB-CBh-hSpCas9] (BioVector Science lab, China) via BbsI restriction enzyme sites upstream of the scaffold gRNA sequence. A mixture of transcribed Cas9 and gRNA was microinjected into Wistar rat zygotes. After microinjection, the injected zygotes were transferred to pseudopregnant Wistar rats. All rats used in this study were bred in an AAALAC-accredited facility with ad libitum access to food and water. All animal experiments were approved by the Animal Research Ethics Committee of Shanghai Jiaotong University Affiliated Sixth People’s Hospital.

Genomic DNA preparation and genotyping

Genomic DNA was extracted from the tails of 7-day-old rats. In brief, each rat tail was dissolved in 400 ul SNET (100 mM Tris-Cl, 25 mM EDTA, 100 mM NaCl, 0.5% SDS, 0.1 mg/ml Proteinase K) at 55°C for overnight. The genomic DNA was extracted in the lysis mixture by adding 2.5 volumes of alcohol, and dissolved in
200ul ddwater at 55°C for at least 4h. The PCR primers used for genotyping the modified rats are as follows: UOX-forward primer: 5’ - CCCAGGCTAAACTCTCA GGCT-3’; reverse primer: 5’ -TGTCAGGGAAACAGTCATTTCACA-3’. PCR reactions (initial denaturation at 93°C for 3 minutes; 40 cycles at 93°C for 30 seconds, 57°C for 30 seconds, and 65°C for 2 minutes, and a final extension at 65°C for 10 minutes) were performed using Multiplex PCR Mix (novoprotein, China) in an Applied Biosystems Veriti™ 96-Well Thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA). The PCR products were used for sequencing analysis and the sequencing primer was UOX-forward primer.

**Observation of serum uric acid level and renal function**

WT and UOX-KO male and female rats (n=5 per group) were used to analysis the serum uric acid level and kidney function. Rats were fasted overnight every 4 weeks from 4 weeks of age to 52 weeks of age. Blood was collected from the tail vein on the next morning and then incubated at room temperature for 1 hour. Serum were collected and stored at -80 degrees for biochemical indicators analysis. Urine sample for each mouse in 24 h was collected in a metabolic cage, and centrifuged at 2000 × g for 10 min to remove the particulate contaminants. The supernatant was used for the determination of uric acid, creatinine, and protein levels.
**Biochemical indicators analysis**

Biochemical indicators including serum UA, creatinine and BUN were determined using corresponding kits (Nanjing Jiancheng Bioengineering Institute, China). 24-h urine was collected for urine creatinine and uric acid detection by using automatic biochemical analyzer (Toshiba, Tokyo, Japan). Urine protein was measured by BN2 automatic protein analyzer (Siemens, Germany).

**Drug treatment**

Five male WT and ten male UOX-KO rats were used to test the effect of autophagy on uric acid nephropathy. 10 male UOX-KO rats were randomly divided into two groups: blank and 3-MA (15mg/kg/day)(MCE, China), respectively. WT and UOX-KO blank group were given normal saline. The drugs and normal saline were given intragastric continuously every 24 h for 4 weeks. Fasting serum were collected every week for biochemical indicators analysis. From 24 hour before final administration, urine sample for each rat in 24 hour was collected. At the end of treatments, kidney tissues were collected for histology or stored at -80 degrees for western blot analysis.

**Western blotting**

Livers or kidneys were lysed with the RIPA buffer (Beyotime Biotechnology, China) with 100 μM PMSF and a phosphatase inhibitor cocktail (Roche). Homogenates were centrifuged at 12,000 g for 15 min, and 40μg of supernatants protein were separated by standard SDS-PAGE and transferred to PVDF membranes.
Blocking was performed with 5% defatted dry milk for 1 h at room temperature. In general, primary antibodies were obtained from CST (Cell Signaling Technology) and used at 1:1,000 in TBST containing 5% BSA. Those antibodies were anti-Atg3 (#3415), Atg7 (#8558), Atg16L1 (#8089), Beclin-1 (#3495), LC3A/B (#12741), p62 (#39749), p-AMPK (#2535), AMPK (#2532), p-JNK (#4668), JNK (#9525), p-ERK (#4370), ERK (#4695), p-mTOR (#2974), mTOR (#2983) and β-tubulin (#2128). The exception was anti-uricase (1:500, sc-166214, Santa Cruz Biotechnology), caspase-1 p10 (1:500, sc-514, Santa Cruz Biotechnology), NLRP3 (1:1000, NBP2-12446, Novus) and IL-1β (1:2000, AF-401-NA, R&D systems). After extensive washes, secondary antibodies HRP linked (CST) were used at 1:5,000 dilution. Specific proteins were visualized using ECL chemiluminescent substrate (Millipore). All results were normalized against β-tubulin, with each measurement performed in triplicate.

Renal histology, immunohistochemistry, crystal detection and transmission electron microscopy

Kidneys were embedded in paraffin and sliced into 4-mm thick sections, stained by hematoxylin-eosin and Masson’s trichrome for histological analysis. Immunohistochemical detection of inflammation was performed with primary antibodies against macrophage marker F4/80 (1:200, #30325, CST), NLRP3 (1:200) and IL-1β (1:200). Renal fibrosis was detected using collagen 1 (1:200, CST) and α-SMA (1:200, rabbit, 19245, CST) staining. Renal crystal sections were also
obtained from absolute ethanol-fixed kidneys to detect uric acid crystals under polarized light. For transmission electron microscopy, kidneys were fixed with 2.5% glutaraldehyde, and post-fixed in 1% phosphate-buffered osmium tetroxide. After being embedded, sectioned, and double-stained with uranyl acetate and lead citrate, images were captured with a transmission electron microscope (Tecnai G2 20 200kV, FEI, USA).

Statistics

Values are expressed as means ± SD. All groups of animals were studied in parallel. Comparisons between different groups were performed by Student’s t-test for unpaired samples. The level of significance was \( P < 0.05 \). These analyses were conducted by using SPSS 21.0 and the Prism 6 software package (GraphPad, San Diego, CA, USA).
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Competing interests

The authors have declared that no competing interest exists.

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Data and reagent availability

UOX-KO model can be requested by contacting the corresponding authors.

Author contributions statement

M.W. S.Q. and H.B.C. designed experiments and wrote the manuscript. M.W., Y.C.M., X.T.C and N.L. performed experiments. M.W., Y.W.M., and X.T.C. performed analysis and interpretation of data.
References


Figure 1. Generation of UOX-knockout (KO) rats with hyperuricemia

(A) Schematic representation of UOX KO using CRISPR/Cas9. Boxes indicate exons, and horizontal lines indicate introns (above), the UOX gene editing site (grey), and protospacer adjacent motif (PAM) region (black). (B) Sanger sequencing of the polymerase chain reaction-amplified genomic UOX locus revealed DNA disruption at the gRNA target site downstream of the Cas9 cleavage site in heterozygous (HE), but not in wild-type (WT) or homozygous (HO) rats. (C) Schematic representation of UOX-locus targeting and deep sequencing reads with deletions (red boxes) in HO rats. (D) Hepatic expression of UOX protein determined by Western blotting (8-week-old males, n = 5). (E) Serum urate levels of WT, HE, and HO rats of both sexes (8 weeks of age, n = 5 per group). (F) Body weights of WT and HO rats of both sexes from 4 to 52 weeks of age (n = 5 per group). (G) Serum urate levels of WT and HO rats of both sexes.
sexes from 4 to 52 weeks of age ($n = 5$ per group). (H) Survival rates of HO (male, $n = 50$; female, $n = 50$) vs. WT rats (male, $n = 50$; female, $n = 50$) determined using Kaplan–Meier curves from birth to 56 weeks of age. Values are presented as the means ± standard deviations (SD). *$P < 0.05$, ***$P < 0.001$. 
Figure 2. Renal function was impaired in UOX-KO rats

Serum creatinine (A) and urea nitrogen (B) in UOX-KO vs. WT rats of both sexes. Levels of 24-h urine volume (C), urine protein (D), urine uric acid (E), and urine creatinine (F) in UOX-KO and WT rats of both sexes at 8 weeks of age (n = 5 per group). Values are presented as the means ± SDs. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 3. Renal histopathology was impaired in *UOX*-KO rats

(A) Complete kidney and longitudinal sections of male *UOX*-KO and WT rats aged 4–52 weeks. Black arrows indicate lesions in 4 and 8 weeks of age, and urate crystals in 24 and 52 weeks of age in UOX-KO rats. (B) Urate crystals in the kidney were detected under polarized light. (C) Transmission electron microscopy of samples from male WT and *UOX*-KO rats at 8 weeks of age. Blue arrows indicate podocytes and the red arrow indicates the basement membrane. (D) Hematoxylin and eosin (H&E) staining of kidneys from *UOX*-KO rats and WT controls. (E) Masson’s trichrome staining of kidneys from *UOX*-KO rats and WT controls. Renal expression of collagen I (F), alpha-smooth muscle actin (α-SMA) (G), F4/80 (H), and interleukin (IL)-1β (I) determined by immunostaining with corresponding antibodies (*n* = 5 per group). Black arrows in E-I indicate positive staining. Tissues in (D–I) were collected from 12-week-old rats. Scale bars = 0.5 cm (A), 200 μm (B), 2μm (C), 50 μm (D–I).

Values are presented as the means ± SDs. *P* < 0.05, **P** < 0.01, ***P*** < 0.001.
Figure 4. Hyperuricemia increased autophagy in the kidney

(A) Samples from 12-week-old male WT and UOX-KO rats were examined using scanning electron microscopy. Autophagosomes are indicated by red arrows. (B) Renal expression of autophagy-related proteins determined by Western blotting. (C) Renal expression of phosphorylated and total AMPK, JNK, ERK, and mTOR proteins determined by Western blotting. Scale bars = 1μm (A). Data in (B and C) are shown as the means ± SDs and are representative of at least three independent experiments.

*P < 0.05, **P < 0.01, ***P < 0.001.
Figure 5. Inhibition of autophagy prevented renal dysfunction and alleviated renal histopathological changes in UOX-KO rats

Rats were sacrificed at 4 weeks after 3-MA administration. Body weight (A), fasting serum uric acid (B), creatinine (C), and blood urea nitrogen (BUN) (D) levels were measured weekly. The 24-h urine volume (E), urine protein (F), urine uric acid (G), and urine creatinine (H) levels were measured at the end of the experiments. (I) Renal histopathology was determined using H&E and Masson’s trichrome staining. Renal expression of collagen I, α-SMA, F4/80, NLRP3, and IL-1β was determined by
immunostaining with corresponding antibodies. (J) Renal expressions of autophagy-related proteins and upstream pathways including AMPK, ERK and JNK were determined by Western blotting. (K) Renal expressions of NLRP3 inflammasome related proteins. Black arrow indicate renal tubules dilation in H&E staining, and positive staining in Masson, collagen I, α-SMA, F4/80, NLRP3, and IL-1β staining. Data in J and K are representative of four independent experiments. (n = 5 per group; A–I). Scale bar = 50 μm. Values are presented as the means ± SDs. *P < 0.05, **P < 0.01, ***P < 0.001.