

Glucocorticoids increase renal excretion of urate in mice by down-regulating urate transporter 1

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Running title: Glucocorticoid receptor regulates uric acid homeostasis

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Abbreviations:

DEX, dexamethasone; DIC, diclofenac; IBU, ibuprofen; INDO, indomethacin; MIF, mifepristone; NSAIDs, nonsteroidal anti-inflammatory drugs; PCN, pregnenolone-16 α -carbonitrile; siRNA, small interfering RNA; TA, triamcinolone acetonide; XDH, xanthine dehydrogenase; XOD, xanthine oxidase; XOR, xanthine oxidoreductase.

Abstract:

Both non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids have been widely used for the treatment of gout, a disease promoted by excess body burden of uric acid (UA). However, their effects on the homeostasis of UA remain poorly understood. The present study showed that 1-week treatments with three NSAIDs (ibuprofen, diclofenac and indomethacin) had little effect on UA homeostasis in mice, whereas 1-week low doses (1 and 5 mg/kg) of dexamethasone (DEX) significantly decreased serum UA about 15%. Additionally, low doses of DEX also resulted in an increase in hepatic UA concentration and urinary UA excretion, which were associated with an induction of xanthine oxidoreductase (XOR) in liver and a down-regulation of urate transporter 1 (Urat1) in kidney, respectively. Neither 75 mg/kg DEX nor 100 mg/kg pregnenolone-16 α -carbonitrile (PCN) altered UA concentrations in serum and livers of mice, suggesting that the effect of DEX on UA homeostasis was not due to the pregnane X receptor (PXR) pathway. Further *in vitro* studies demonstrated that glucocorticoid receptor (GR) was involved in DEX-mediated down-regulation of Urat1. Knockdown of both p65 and c-Jun completely blocked the effect of DEX on Urat1, suggesting that GR regulates Urat1 via its interaction with both nuclear factor kappa B (NF- κ B) and activator protein 1 (AP-1) signaling pathways. To conclude, the present study identifies for the first time a critical role of glucocorticoids in regulating UA homeostasis,

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and elucidates the mechanism for GR-mediated regulation of Urat1 in mice.

Significance Statement : This study demonstrates for the first time a critical role of glucocorticoid receptor in regulating urate transporter 1 in mouse kidney.

Introduction

Gout, a chronic disease triggered by the deposition of monosodium urate crystals in the joints, has become the most common type of inflammatory arthropathy in adults with a general prevalence of 1-4% (Dalbeth et al., 2016). The central pathological feature of gout is the elevated concentrations of serum uric acid (UA), also known as hyperuricemia, caused by an imbalance of UA production and elimination from the body. UA is mainly synthesized in the liver during the metabolism of both endogenous (DNA, RNA, and ATP) and exogenous (dietary) purines. Xanthine oxidoreductase (XOR), which exists in the form of either xanthine dehydrogenase (XDH) or xanthine oxidase (XOD), regulates the rate-limiting step, catalyzing the conversion from hypoxanthine to xanthine and from xanthine to UA (Maiuolo et al., 2016). It should be noted that UA is the final product of purines in humans, whereas it is further converted to more soluble allantoin by uricase in most other species (Kratzer et al., 2014).

Elimination of UA occurs via two major routes, with approximately 70% excreted through the kidney and the remaining 30% through the intestine (Sorensen, 1965). UA in the circulation is freely filtered at the glomeruli, and about 90% of the filtered UA is reabsorbed into the blood (Mount, 2005). Various transporters are involved in the UA disposition in the kidney. Among them, urate transporter 1 (URAT1), an urate/anion exchanger at the apical membrane of the proximal tubule, is one of the two most critical transporters

for renal reabsorption of UA. The association between URAT1 and hyperuricemia has been widely established in many genetic studies (Tin et al., 2018). In contrast, studies on the regulation of URAT1 are quite limited. URAT1 has been reported to be regulated by insulin and alpha kinase 1 (Kuo et al., 2017; Toyoki et al., 2017). Nonetheless, the exact regulatory mechanisms of URAT1 expression remain elusive.

Both nonsteroidal anti-inflammatory drugs (NSAIDs) and steroids have been widely used for pain relief and inflammation suppression during an acute gout attack (Ragab et al., 2017). Aspirin was reported to alter the renal excretion of UA by either cis- or trans-inhibition of URAT1 (Enomoto et al., 2002; Zhang et al., 2014). Glucocorticoids, such as dexamethasone (DEX), have been shown to increase xanthine oxidase activity in rats (Patel et al., 2014). Nevertheless, the effect of glucocorticoids and NSAIDs other than aspirin on UA homeostasis is largely unknown. The present study aimed to systematically investigate the effect of NSAIDs and DEX on UA homeostasis.

Materials and methods

Chemical reagents

DEX, UA, XOD, and pregnenolone-16 α -carbonitrile (PCN) were purchased from Sigma-Aldrich (St. Louis, MO). Triamcinolone acetonide (TA) was obtained from Energy Chemical (Shanghai, China) and mifepristone (MIF) was purchased from Macklin (Shanghai, China). Ibuprofen (IBU), diclofenac (DIC), and indomethacin (INDO) was purchased from Alfa Aesar (Ward Hill, UK).

All other chemicals were commercial products of the highest purity grade available.

Animals and treatments

All animal studies were carried out at the Institute of Radiation Medicine of the Chinese Academy of Medical Sciences (CAMS, Tianjin, China) and approved by the Institutional Animal Care and Use Committee at the CAMS. Adult male C57/BL6 (20±2 g) mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). Mice were housed in an environmentally controlled breeding room (temperature: 22±2 °C, humidity: 55±5%, and 12-hr light/dark cycle) and water *ad libitum*.

IBU has been recommended for use as a pain reliever in mice, with a ranging dose of 7.5 to 30 mg/kg (Jenkins, 1987; Liles and Flecknell, 1992). In this study, we chose the maximal recommended dose of IBU (30 mg/kg). Previous studies in mice suggested that oral treatments with 2.5-5 mg/kg DIC or 1 mg/kg INDO were sufficient to exert their anti-inflammation and analgesic effects in mice (Fujiyoshi et al., 1989; Mun et al., 2009; Ritesh et al., 2016). However, 10 mg/kg DIC or INDO was shown to induce marked adverse effects in the GI tract of mice (Harusato et al., 2009; Yamada et al., 2011; Akingbasote et al., 2016; Xiao et al., 2017). In our preliminary study, mice were orally gavaged with DIC (5 and 10 mg/kg) or INDO (2.5 and 7.5 mg/kg) to evaluate their potential toxicities. The data showed that both 10 mg/kg DIC and 7.5 mg/kg INDO markedly decreased body weights and increased liver and spleen

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weights (as determined via the tissue : body-weight ratios), with marked alterations in liver and intestine morphology (Supplemental Figure 1). In contrast, both 5 mg/kg DIC and 2.5 mg/kg INDO were well tolerated by mice. Therefore, we chose 5 mg/kg DIC and 2.5 mg/kg INDO for the present study. Additionally, several NSAIDs are reported to regulate the expression of a target gene, namely NSAID-activated gene-1 (NAG-1), which plays an important role in cell growth and proliferation (Kim et al., 2002). As shown in Supplemental Figure 2, the mRNA expression of NAG-1 was induced in livers of mice treated with 30 mg/kg IBU, 5 mg/kg DIC, and 2.5 mg/kg INDO (Supplemental Figure 2). Therefore, our data suggests that the dose of each NSAID is sufficient to exert its therapeutic effect and relatively close to the maximum non-toxic dose. Mice were randomly divided into 4 groups (N=5): Control, IBU (30 mg/kg, p.o.), DIC (5 mg/kg, p.o.), and INDO (2.5 mg/kg, p.o.). The doses and schedules of NSAIDs were selected according to the previous studies (Wang et al., 1994; Klopčič et al., 2008; Akingbasote et al., 2016). NSAIDs were dissolved in sterile saline containing 0.25% sodium carboxymethylcellulose and 0.5% Tween-80. Mice were treated orally with NSAIDs once daily for 7 days. To investigate the effects of DEX on UA homeostasis, mice were treated with DEX at either 1 or 5 mg/kg (DEX-1 and DEX-5) intraperitoneally for 7 days. DEX was dissolved in sterile saline containing 10% PEG400. To determine the effect of PXR activation on UA homeostasis, mice were treated intraperitoneally with either DEX (75 mg/kg;

DEX-75) or PCN (100 mg/kg) for 4 days. The vehicle control for the high-dose DEX and PCN was corn oil.

Urine and tissue sample collection

Urine was collected for three 24-hr periods on 2nd, 4th, and 6th day from each mouse using individual metabolic cages. Urine samples were centrifuged at 3,000 × g at 4 °C for 10 min to collect the supernatants for further analysis. On the last day of the study, blood was collected by orbital bleeding 1 hr after the corresponding administrations, and centrifuged at 5,000 × g at 4 °C for 20 min to obtain serum. Kidneys and livers were harvested, rinsed, snap frozen in liquid nitrogen, and stored at -80 °C.

Quantification of UA

Serum and urine UA concentrations were quantified using a standard diagnostic kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instruction. Liver UA concentration was quantified using HPLC analysis. Briefly, liver tissue was homogenized in 2 volumes of ddH₂O, and 40 µl of homogenate was gently mixed with 120 µl of 0.2 M perchloric acid containing 1,3-dimethyl uric acid (40 µM) as the internal standard. The mixture was centrifuged at 20,000 × g for 10 min and 20 µl of the supernatant was injected for HPLC analysis. HPLC was performed on a Agilent 1260 infinity II with a Zorbax SB-C18 (2.1 × 150 mm, 5 µm) column (Agilent Technologies, Santa Clara, CA). Mobile phase A contained 0.1% formic acid, and mobile phase B was acetonitrile. The entire process of separation was went on a

gradient elution at a flow rate of 0.5 ml/min for 15 min: initiated with 8% B for 2 min, increased to 20% B in 1 min, and further increased to 90% B in 7 min, kept 90% B for 1 min, decreased to 8% B in 1 min, and balanced for 3 min before the next injection. Before the injection, the needle was washed with 50% methanol for 5 s, and the injection volume was 20 μ l. Detection wavelength for UA was 294 nm (Tsushima et al., 2013).

Assays for XOD and uricase activities

Liver tissue was homogenized in 9 volumes of 50 mM KH_2PO_4 buffer containing 0.5 mM EDTA (pH 7.4) in an ice bath. The homogenate was centrifuged at 4,000 \times g for 20 min at 4 $^\circ\text{C}$, and the lipid layer was carefully discarded. The supernatant was further centrifuged at 10,000 \times g for 10 min at 4 $^\circ\text{C}$ and the final supernatant was collected for the assays. Protein concentration of the final supernatant was determined with the bicinchoninic acid (BCA) method according to the manufacturer's instructions (CW BIO, Beijing, China). To determine the XOD activity, XOD standard solutions were prepared by diluting XOD stock in 50 mM KH_2PO_4 buffer containing 0.5 mM EDTA (pH 7.4). Subsequently, a mixture of 25 μ l substrate solution (1.5 mM xanthine, 1.0 mM potassium oxonate in 0.02 M NaOH), 25 μ l liver extract or XOD standard solution, 150 μ l KH_2PO_4 buffer (50 mM) were loaded into a 96-well plate, and incubated at 37 $^\circ\text{C}$ for 30 min. The absorbance of UA production was read at 294 nm. To determine uricase activity, liver extract was incubated with 30 μ M UA in 50 mM borate buffer (pH 9.0) at 37 $^\circ\text{C}$ for 30 min.

The decrease of UA was determined by the decrease of absorption at 294 nm.

Cell culture and treatment

Normal rat kidney epithelial cells (NRK-52E) were obtained from cell resource center of Shanghai Institutes for Biological Sciences (Shanghai, China). Cells were cultured with DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in 5% CO₂. The chemicals were dissolved in dimethyl sulfoxide. Cells were seeded in triplicate in 24-well plates at a density of 2.5×10^5 /well and allowed to attach overnight. Twenty-four hours after plating, DEX, TA and MIF diluted in fresh DMEM containing 5% FBS were added. The final concentrations of DEX, TA and MIF were 1, 0.5 and 0.15 µM, respectively, as previously described (Bradley and Mishell, 1981; Ray and Prefontaine, 1994; Boonyaratanakornkit et al., 1998). Cells were harvested either 24 hr (RNA isolation) or 48 hr (protein extraction) later for further analysis.

Gene silencing with small interfering RNAs

Small interfering RNAs (siRNAs) were synthesized by Genepharma (Suzhou, China). siRNAs were designed according to previous studies, and the sequences were listed in Supplemental Table 1 (Numakawa et al., 2009; Sirabella et al., 2009; Ban et al., 2011). siRNAs were transfected into NRK-52E cells using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, about 12-24 pmol siRNAs and 1 µl of Lipofectamine were diluted in 100 µl Opti-MEM, mixed gently and

then incubated for 20 min at room temperature. The entire mixture was added to the well of a 24-well plate and then NRK-52E cells were seeded at a density of 2.5×10^5 /well in DMEM medium with 5% FBS. The mixture was incubated with cells for 24 hr, followed by various treatments for indicated time periods.

RNA isolation and qRT-PCR analysis

Messenger RNA (mRNA) was isolated using Trizol (Takara, Dalian, China) according to the manufacturer's protocol. After isolation, cDNA was synthesized with a High Capacity cDNA Archive kit (Vazyme, Nanjing, China). The PCR reactions were performed on an ABI QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems, Foster City, CA) using Ultra SYBR Mixture (CW BIO, Beijing, China). The comparative threshold cycle (Ct) method was used to quantify the fold change ($2^{-\Delta\Delta C_t}$) for various mRNAs, which were normalized to 36b4 mRNA and presented as relative fold change to the control group. Primer sequences used in this study are summarized in Supplemental Table 2.

Western blot

Total protein was isolated using RIPA lysis buffer (CW BIO, Beijing, China) according the manufacturer's protocol. Equal amounts of protein samples (40 μ g) were separated by electrophoresis on 10% SDS-polyacrylamide gels and then transferred to PVDF membranes. The membranes were blocked with 5% skim milk followed by overnight incubation with primary antibodies against p65 (1:1000, #8242, Cell Signaling Technology, Danvers, MA), c-Jun (1:1000,

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#9165, Cell Signaling Technology, Danvers, MA), URAT1 (1:500, #250521, Abbiotec, San Diego, CA) and β -Actin (1:1500, K101527P, Solarbio, Beijing, China). Blots were then further incubated with secondary antibody (1:5000, sc-2004, Santa Cruz, Dallas, TX) in 5% skim milk, and signals were visualized with ECL reagents (Merck Millipore, Darmstadt, Germany). Images were visualized by the Amersham Imager 600 (GE Healthcare, Pittsburgh, PA) and analyzed using Image J software (National Institutes of Health, Bethesda, MA).

Data analysis and statistics

Data were analyzed using GraphPad Prism software version 7.0. (GraphPad, San Diego, CA), and expressed as means \pm SEM. The differences between groups were analyzed using a one-way analysis of variance (ANOVA) followed by two-by-two comparisons using Tukey's test. P value < 0.05 was considered significant.

Results

DEX, but not NSAIDs, markedly altered the UA homeostasis in mice. As shown in Figure 1A and 1B, three NSAIDs had no effect on UA concentrations in serum or livers of mice. Additionally, the activities of XOD and uricase, as well as the mRNA levels of key enzymes involved in UA synthesis were not altered by NSAIDs treatments (data not shown). In contrast, both 1 and 5 mg/kg doses of DEX significantly decreased serum UA (15~19% \downarrow) but significantly increased hepatic UA (29~36% \uparrow), as compared to control mice (Figure 1C and 1D). Further analysis demonstrated that both doses of DEX

significantly increased urine volume collected for 24 hr on the 2nd (69% and 100%↑), 4th (90% and 170%↑) and 6th (50% and 69%↑) day of treatment (Figure 1E). Additionally, as shown in Figure 1F, DEX also significantly increased the 3-day cumulative UA excretion (56% and 77%↑ for 1 and 5 mg/kg, respectively).

DEX increased UA synthesis in livers of mice via XOR induction. The mRNA levels of key enzymes involved in UA synthesis were quantified to investigate the mechanism for DEX-mediated increase of UA in livers of mice. DEX had no effect on the mRNA levels of phosphoribosyl amidotransferase (Ppat), phosphoribosyl pyrophosphate synthetase (Prps), adenosine deaminase (Ada) and hypoxanthine-guanine phosphoribosyltransferase (Hprt) (Figure 2A). UA in livers of mice is mainly regulated by the activities of XOD (key enzyme in UA synthesis) and uricase (enzyme for UA oxidization). DEX significantly increased Xdh mRNA levels in a dose-dependent manner (26% and 110%↑ for 1 and 5 mg/kg, respectively), whereas it had no effect on uricase mRNA levels. Consistently, both doses of DEX significantly increased XOD activity (61% and 160%↑), whereas they did not alter uricase activity in livers of mice (Figure 2B and 2C). To conclude, the current data suggests that DEX has an inductive effect on Xdh mRNA expression and thus increases UA synthesis in livers of mice.

DEX decreased both mRNA and protein levels of Urat1 in kidneys of mice.

Organic anion transporter 1 and 3 (Oat1 and 3) are basolateral UA uptake

transporters. DEX had no effect on the mRNA expression of Oat1, but significantly decreased the mRNA expression of Oat3 (52% and 81%↓ for 1 and 5 mg/kg, respectively) in a dose-dependent manner (Figure 3A). Breast cancer resistance protein (Bcrp), multidrug resistance protein 4 (Mrp4), as well as sodium-dependent phosphate cotransporter type 1 and 4 (Npt1 and 4) are apical UA efflux transporters involved in renal secretion of UA. DEX did not alter Bcrp or Mrp4 mRNA levels, whereas it significantly increased the mRNA levels of Npt1(72% and 47%↑) and 4(99% and 65%↑ for 1 and 5 mg/kg, respectively). Oat10, glucose transporter 9 (Glut9), and Urat1 are apical UA uptake transporters that play a critical role in UA reabsorption. DEX showed no effect on Oat10 and Glut9, but significantly decreased Urat1 mRNA levels (40% and 44%↓ for 1 and 5 mg/kg, respectively) in a dose-dependent manner (Figure 3B). Consistently, both doses of DEX significantly decreased the protein levels of Urat1 in kidneys of mice (37% and 45%↓) (Figure 3C). Therefore, the current data suggests that DEX appears to increase UA secretion (Npt1 and 4) and decrease UA reabsorption (Urat1) in kidneys of mice.

DEX-mediated regulation of UA homeostasis was not due to PXR signaling pathway. DEX at higher doses (> 20 mg/kg) is known to activate pregnane X receptor (PXR) in mice (Xie et al., 2000), whereas lower doses (< 6 mg/kg) mainly activate glucocorticoid receptor (GR) signaling (Heuman et al., 1982). Both 1 and 5 mg/kg DEX significantly increased the GR target gene

serum- and glucocorticoid-induced protein kinase 1 (Sgk1) in livers (23-fold and 20-fold \uparrow) and kidneys (12-fold and 14-fold \uparrow) of mice, suggesting the activation of GR signaling pathway (Figure 4A). To determine the effect of PXR activation on UA homeostasis, mice were given either a high dose of DEX (75 mg/kg) or PCN, a well-known PXR agonist. Both 75 mg/kg DEX and PCN significantly increased mRNA levels of the PXR target gene cytochrome P450 3a11 (Cyp3a11) in livers of mice (33-fold and 27-fold \uparrow), suggesting the activation of the PXR pathway (Figure 4B). However, neither 75 mg/kg DEX nor PCN altered the UA concentrations, Xdh mRNA, or XOD activity in livers of mice (Figure 4C-4E). Therefore, GR activation, but not PXR activation, mediates the effect of DEX on hepatic UA levels in mice.

GR regulated DEX-mediated down-regulation of Urat1 *in vitro*. NRK-52E cells were treated with DEX and TA, two GR agonists, to determine the role of GR in Urat1 regulation. The activation of GR by DEX and TA was demonstrated by the increased mRNA levels of Sgk1 (3.3-fold and 5.2-fold \uparrow for 1 and 5 mg/kg, respectively) (Figure 5A). Both mRNA and protein levels of Urat1 were significantly decreased by DEX (mRNA 63% \downarrow ; protein 38% \downarrow) and TA (mRNA 59% \downarrow ; protein 57% \downarrow) (Figure 5B and 5C). Notable, GR antagonist MIF reversed the down-regulating effect of DEX and TA on Urat1 mRNA expression (D+M versus DEX, 97% \uparrow ; T+M versus TA, 77% \uparrow) (Figure 5C). Additionally, GR knockdown by siRNA ablated the down-regulating effect of DEX and TA on Urat1 (Figure 5D). It should be mentioned that both MIF and

GR knockdown significantly increased the mRNA levels of Urat1 (MIF, 47%↑; GR knockdown, 190%↑) (Figure 5C and 5D). Collectively, the current data suggests that GR signaling pathway plays a critical role in regulating Urat1 in kidney cells.

Both NF-κB and c-Jun were involved in GR-mediated regulation of Urat1

in vitro. GR-dependent transcriptional regulation depends on its interaction with other co-regulatory proteins. *In silico* analysis suggest that Urat1 promoter region contained several putative NF-κB and AP-1 binding sites (Table 1). Next, NF-κB/p65 or AP-1/c-Jun was knocked down in NRK-52E cells to investigate the role of NF-κB and AP-1 in Urat1 regulation. Both 20 and 40 nM p65 siRNA significantly decreased mRNA levels of p65 (20 nM, 74%↓; 40nM, 73%↓) and Urat1 (20 nM, 59%↓; 40 nM, 54%↓) (Figure 6A and 6C). Similarly, c-Jun mRNA levels were significantly decreased by both 20 nM (69%↓) and 40 nM (74%↓) c-Jun siRNA treatments (Figure 6B). However, only 40 nM c-Jun siRNA was able to decrease Urat1 mRNA levels (55%↓) (Figure 6C). Thus, 40 nM p65 and c-Jun siRNA were used in the subsequent treatments, showing that neither p65 knockdown nor c-Jun knockdown was able to completely prevent the down-regulating effects of DEX and TA in kidney cells (Figure 6D). To conclude, the current data suggests that both p65 and c-Jun play an important role in maintaining the basal level of Urat1, and are partly involved in GR-mediated down-regulation of Urat1 mRNA expression.

Knockdown of both p65 and c-Jun completely blocked the

down-regulating effect of GR activation on Urat1 *in vitro*. To further investigate the role of p65 and c-Jun on GR-mediated regulation of Urat1, both p65 and c-Jun were knocked down in NRK-52E cells. The decreased mRNA (p65, 71%↓; c-Jun, 75%↓) and protein levels (p65, 85%↓; c-Jun, 80%↓) of p65 and c-Jun validated the knockdown effect of siRNA treatments (Figure 7A and 7B). The knockdown of both p65 and c-Jun completely reversed the down-regulating effect of DEX and TA on both mRNA and protein levels of Urat1 (Figure 7C). Therefore, the current data suggests that GR-mediated regulation of Urat1 is dependent on both p65 and c-Jun.

Discussion

Despite the wide use of NSAIDs and glucocorticoids in gout treatment, their effects on UA metabolism are poorly investigated. Our data suggests that oral administration with high doses of NSAIDs causes adverse effects in both livers and intestine of mice. According to both literature and preliminary studies, we treated mice with each NSAID at a dose which is relatively close to the maximum non-toxic dose, and demonstrated that the three NSAIDs at therapeutic doses had no effect on UA homeostasis. In contrast, DEX significantly altered UA concentrations in mice by increasing both the production and elimination of UA.

XOR is the rate-limiting enzyme catalyzing the conversion from hypoxanthine to xanthine and from xanthine to UA in the liver. Various stimuli, such as hydrogen peroxide, calcium, growth factors and inflammatory

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cytokines, are able to regulate XOR expression and activity (Dupont et al., 1992; Flanders et al., 1997; McNally et al., 2005). Previous studies demonstrated that DEX could induce XOR expression and activity in bovine epithelial cells and rat cremaster muscle (Pfeffer et al., 1994; Wallwork et al., 2003). The current data demonstrate an inductive effect of DEX on both expression and activity of XOR in livers of mice, which was associated with an increase in hepatic UA.

Nuclear receptors play an important role in regulating various physiological processes. Aryl hydrocarbon receptor (AhR) and peroxisome proliferator-activated receptor alpha (PPAR α) have been implicated in regulating XOR (Sugihara et al., 2001; Kanemitsu et al., 2017). PXR is a key regulator of xenobiotic metabolism, and activated by a broad range of endogenous and exogenous chemicals, such as bile acids, steroids and antibiotics (Kliwer et al., 2002). Glucocorticoids are also able to activate the PXR signaling pathway at high doses (Xie et al., 2000). Nonetheless, the present study demonstrated that DEX-mediated induction of XOR was due to the activation of GR, but not PXR. It should be noted that we did not quantify the serum levels of UA, or the mRNA levels of urate transporters in kidneys. Therefore, it could not be excluded that high dose of DEX could still affect the UA distribution. Nonetheless, the present study suggests that PXR activation has no effect on hepatic UA concentrations, Xdh activities, or mRNA levels of UA-related genes (Supplemental Figure 3) in mice.

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The present study demonstrated that DEX significantly increased renal excretion of UA and thus decreased serum UA, despite the increased UA production in the liver. The kidney accounts for about 60-65% of the daily elimination of UA (Sorensen, 1965). UA in the circulation is freely filtered by the glomerulus, and more than 90% of UA is reabsorbed in the proximal tubule. Additionally, tubular secretion of UA also occurs. Both UA reabsorption and secretion involve many membrane transporters (Hyndman et al., 2016). During the tubular secretion, UA is taken up by Oat1 and 3 from blood into kidney, and transported into urine through efflux transporters such as Ntp1, Npt4, Mrp4 and Bcrp. Although DEX increased Ntp1 and 4, it markedly decreased Oat3 in kidneys of mice, suggesting a mixed effect of DEX on UA secretion. Nonetheless, compared to UA reabsorption, the tubular secretion is only a minor component of urine UA excretion. Among three transporters (Urat1, Glut9 and Oat10) involved in UA reabsorption, only Urat1 was significantly decreased by DEX. Therefore, DEX-mediated increase in urine UA excretion is mainly due to its down-regulating effect on Urat1.

Urat1 is considered the most effective transporter in the reabsorption of UA, and its abundance is closely linked to serum UA concentration (Kottgen et al., 2013). Although Urat1 has become the therapeutic target for lowering UA, its regulatory mechanism remains largely unknown. In the present study, GR agonist decreased, whereas GR antagonist or knockdown increased Urat1. Additionally, GR knockdown completely blocked the inhibitory effect of DEX on

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Urat1, suggesting a critical role of GR in regulating Urat1. *In silico* analysis reveal that Urat1 promoter has several putative NF- κ B and AP-1 binding sites. Previous studies reported that the direct interaction between NF- κ B and GR mediated the repression of interleukins by DEX (Ray and Prefontaine, 1994). Similarly, a direct interaction between GR and c-Jun was also reported in previous studies (Schule et al., 1990; Yang-Yen et al., 1990). In the present study, either p65 or c-Jun knockdown markedly decreased Urat1, suggesting an important role of p65 and c-Jun in maintaining the basal level of Urat1. However, only knockdown of both p65 and c-Jun could completely prevent the down-regulation of Urat1 by DEX and TA, suggesting that GR-mediated regulation of Urat1 requires the interaction of both p65 and c-Jun. It is reported that *in vitro* knockdown of GR by RNA interference could enhance the basal NF- κ B activity (Zhu et al., 2004). This may explain the increased basal mRNA level of Urat1 in GR knockdown cells in this study. Therefore, the current data suggests that DEX down-regulates Urat1 through the GR-mediated interaction of both NF- κ B and AP-1.

It should be noted that the present study is limited by considerable species differences in UA metabolism between humans and mice. For instance, UA is metabolized to allantoin by uricase in livers of mice, whereas it is the end product of purine metabolism in humans due to the loss of uricase activity during evolution (Oda et al., 2002). Although transporters such as URAT1, BCRP, OATs, and NPTs are expressed in kidneys of both humans

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and mice, their abilities in handling UA might be different (Hosomi et al., 2012). Nevertheless, the current findings demonstrate for the first time that glucocorticoids play an important role in regulating UA homeostasis via GR signaling pathway. In the liver, GR activation induces XOR expression and activity, and thus elevates UA production. In the kidney, GR activation down-regulates both mRNA and protein levels of Urat1 through NF- κ B and AP-1, and thus enhances UA excretion in the urine.

Authorship Contribution

Participated in research design: Gentao Li, Yuanfu Lu and Youcai Zhang;

Conducted experiments: Gentao Li, Ruicong Ma, Khawar Saeed and Hui Xiong;

Contributed new reagents or analytic tools: Lifeng Han and Youcai Zhang;

Performed data analysis: Gentao Li, Lifeng Han and Youcai Zhang;

Wrote or contributed to the writing of the manuscript: Gentao Li, Curtis D. Klaassen, Yuanfu Lu, and Youcai Zhang.

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Footnotes

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Figure Legends

Figure 1. DEX, but not NSAIDs, markedly alter UA homeostasis in mice.

Male C57/BL6 mice (N=5/group) were administered NSAIDs (30 mg/kg IBU, 5 mg/kg DIC, and 2.5 mg/kg INDO, p.o.) or DEX (1 and 5 mg/kg, i.p.) for 7 days. The UA concentrations were quantified in serum and livers of mice treated with either NSAIDs (A and B) or DEX (C and D). Urine from DEX-treated mice were collected on 2nd, 4th and 6th day, respectively. The urine volume for each 24-hr period was measured. UA concentrations in the urine were quantified, and urinary excretion of UA was calculated for 24-hr periods (E). The total UA in the urine over three days were also calculated (F). Data are expressed as mean \pm SEM (N=5). **P* < 0.05 versus control (Ctrl) group, ****P* < 0.001 versus control (Ctrl) group.

Figure 2. DEX increased UA synthesis in livers of mice via XOD induction.

Male C57/BL6 mice (N=5/group) were administered DEX (1 and 5 mg/kg, i.p.) for 7 days. The mRNA levels of Ppat, Prps, Ada, Hprt, Xdh, and Uricase (A) in livers of mice were quantified using RT-PCR. The activities of XOD (B) and uricase (C) were quantified in livers of mice. Data are expressed as mean \pm SEM (N=5). **P* < 0.05 versus control (Ctrl) group, ***P* < 0.01 versus control (Ctrl) group, ****P* < 0.001 versus control (Ctrl) group.

Figure 3. DEX decreased both mRNA and protein levels of Urat1 in kidneys of mice. Male C57/BL6 mice (N=5/group) were administered DEX (1

and 5 mg/kg, i.p.) for 7 days. The mRNA levels of Oat1, Oat3, Bcrp, Mrp4, Npt1, Npt4, Oat10, and Glut9 (A) were quantified in kidneys of mice. The mRNA (B) and protein levels (C) of Urat1 in kidneys of mice were quantified using RT-PCR and western blot, respectively. Data are expressed as mean \pm SEM (n=5). * P < 0.05 versus control (Ctrl) group.

Figure 4. DEX-mediated regulation of UA homeostasis was not due to PXR signaling pathway. Male C57/BL6 mice (N=5/group) were administered DEX (1 and 5 mg/kg, i.p.) for 7 days. The mRNA expression of Sgk1 was quantified in livers and kidneys (A) of mice. Male C57/BL6 mice (N=5/group) were administered either 75 mg/kg DEX or 100 mg/kg PCN for 4 days. The mRNA levels of Cyp3a11 (B) and Xdh (D) were quantified in livers of mice. UA concentrations in livers of mice were quantified (C), and the XOD activities were also measured (E). Data are expressed as mean \pm SEM (N=5). ** P < 0.01 versus control (Ctrl) group, *** P < 0.001 versus control (Ctrl) group.

Figure 5. GR regulated DEX-mediated down-regulation of Urat1 *in vitro*. NRK-52E cells were incubated DEX, TA and MIF at 1, 0.5 and 0.15 μ M, respectively. Cells were harvested after either 24 hr for RNA isolation or 48 hr for protein extraction. The mRNA levels of Sgk1 (A) and Urat1 (C) were quantified using RT-PCR. The protein levels of Urat1 (B) were determined using western blot. To further determine the role of GR, scramble control and GR-specific siRNAs were transfected into NRK-52E cells using Lipofectamine RNAiMAX. After 24 hr of siRNA transfection, scramble control and

GR-knockdown NRK-52E cells were incubated with 1 μ M DEX or 0.5 μ M TA for additional 24 hr. The mRNA levels of Urat1 were quantified using RT-PCR (D). D+M stands for the combination treatment of DEX and MIF, whereas T+M is the combination treatment of TA and MIF. Data are expressed as mean \pm SEM. * P < 0.05 versus control (Ctrl) group, ** P < 0.01 versus control (Ctrl) group, *** P < 0.001 versus control (Ctrl) group. # P < 0.05 for D+M versus DEX, and T+M versus TA.

Figure 6. Both NF- κ B and c-Jun are involved in GR-mediated regulation of Urat1 *in vitro*. NRK-52E cells were transfected with siRNAs (20 nM and 40 nM) to knock down the mRNA levels of either p65 (A) or c-Jun (B). Their effects on Urat1 mRNA expression (C) were determined. NRK-52E cells were transfected with either p65 siRNAs or c-Jun siRNAs (40 nM) for 24 hr, and then co-treated with either DEX (1 μ M) or TA (0.5 μ M) for additional 24 hr. The mRNA levels of Urat1 were quantified using RT-PCR (D). Data are expressed as mean \pm SEM. * P < 0.05 versus scramble siRNA control. # P < 0.05 versus p65 siRNA control (Ctrl) or c-Jun siRNA control (Ctrl).

Figure 7. Knockdown of both p65 and c-Jun completely blocked the down-regulating effect of GR activation on Urat1 *in vitro*. NRK-52E cells were co-transfected with both p65 and c-Jun siRNAs to knock down their mRNA expression (A). The protein levels of p65 and c-Jun were also quantified using western blot after co-transfection of siRNAs (B). NRK-52E cells were co-transfected with both p65 and c-Jun siRNAs for 24 hr, and then

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were co-treated with either DEX (1 μ M) or TA (0.5 μ M) for additional 24 hr. The mRNA and protein levels of Urat1 were quantified (C). Data are expressed as mean \pm SEM. *P < 0.05 versus scramble siRNA control, **P < 0.01 versus scramble siRNA control, ***P < 0.001 versus scramble siRNA control.

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Table 1. *In silico* analysis of the URAT1 promoter

Species	Predicted elements	Nucleotide positions
Human	AP-1	407—416
		908—917
		967—976
		1154—1163
		1682—1693
		1873—1882
	NF-κB	-353—362
		-548—557
		-1889—1898
Mouse	AP-1	69—78
		137—146
		302—311
		325—334
		547—556
		632—644
		667—676
		1373—1382
		1597—1606
		1750—1759

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		2021—2030

		265—274
		957—966
	NF-κB	1638—1647
		2794—2803

		138—147
		239—248
		303—312
		326—335
		562—571
		893—902
	AP-1	926—935
		1247—1256
		1683—1695
		1733—1742
		1996—2005
		2100—2109

		2402—2411
	NF-κB	2752—2761

Note: The promoters were analyzed using the AliBaba2.1 search tool (<http://gene-regulation.com/pub/programs/alibaba2/index.html>) using default parameters: pairism to known sites 50, matrix width 10 bp, minimum

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number of sites 4, minimum matrix conservation 75%, similarity to sequence
matrix 1%, factor class level 4.

Figure 1

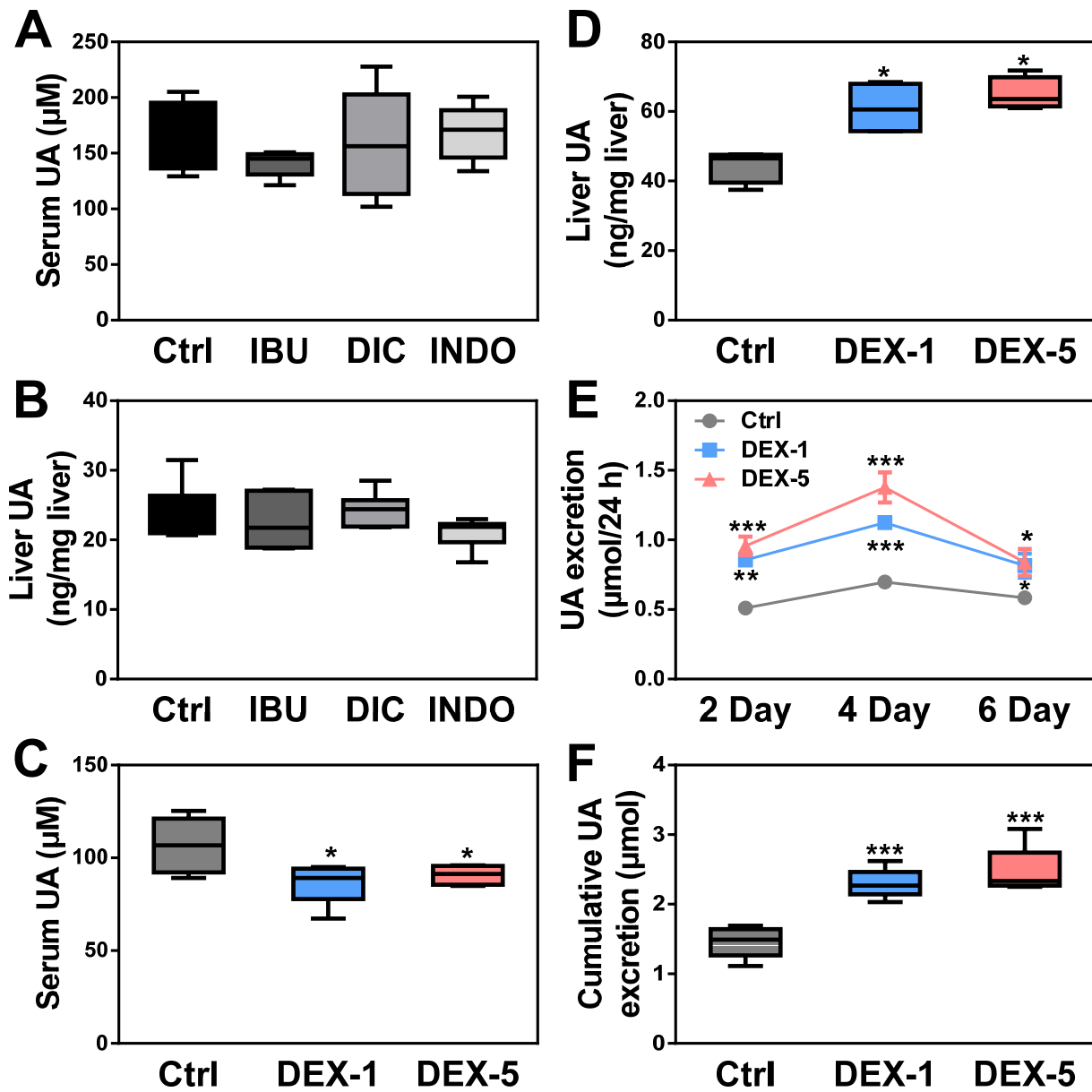


Figure 2

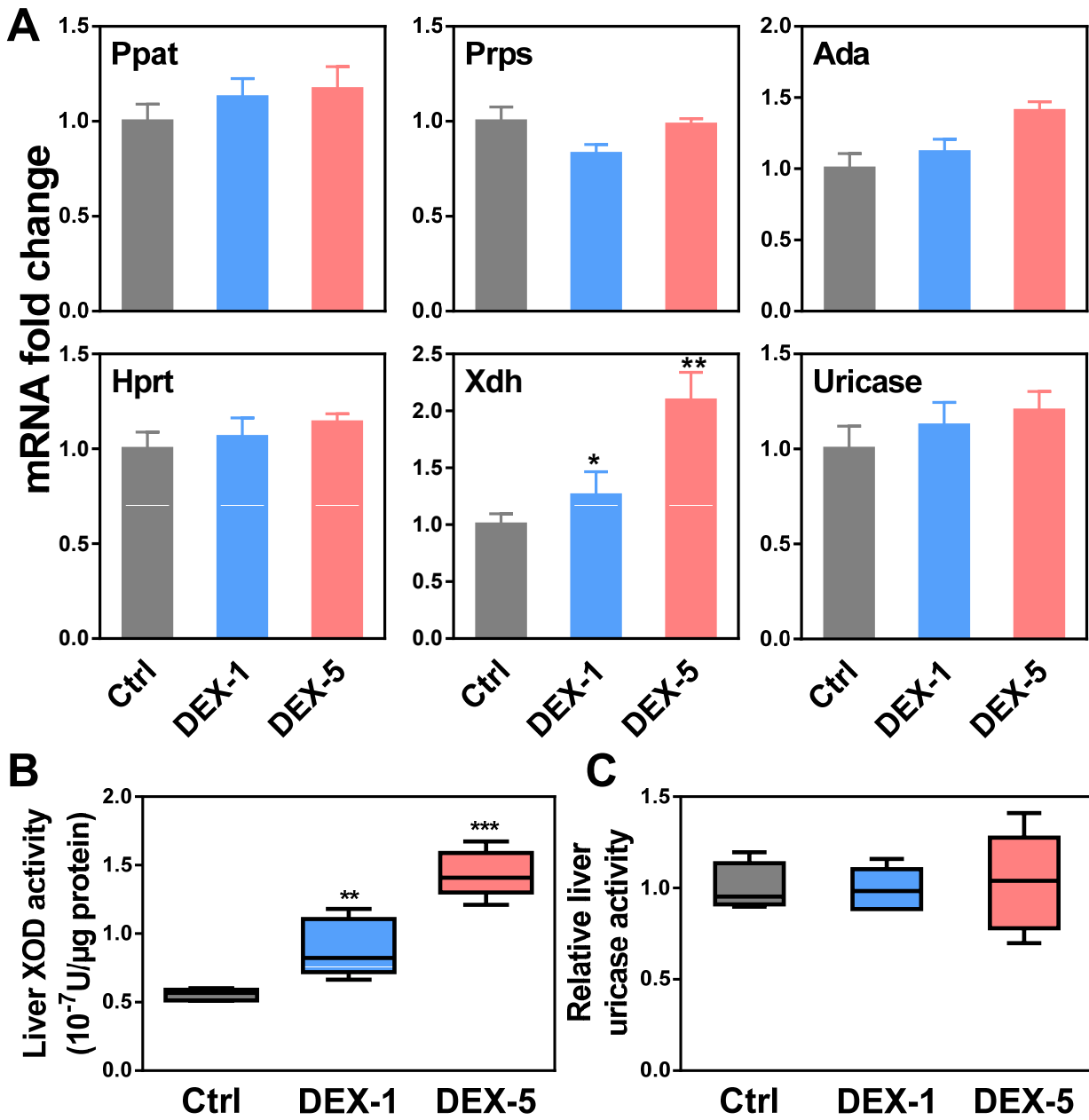


Figure 3

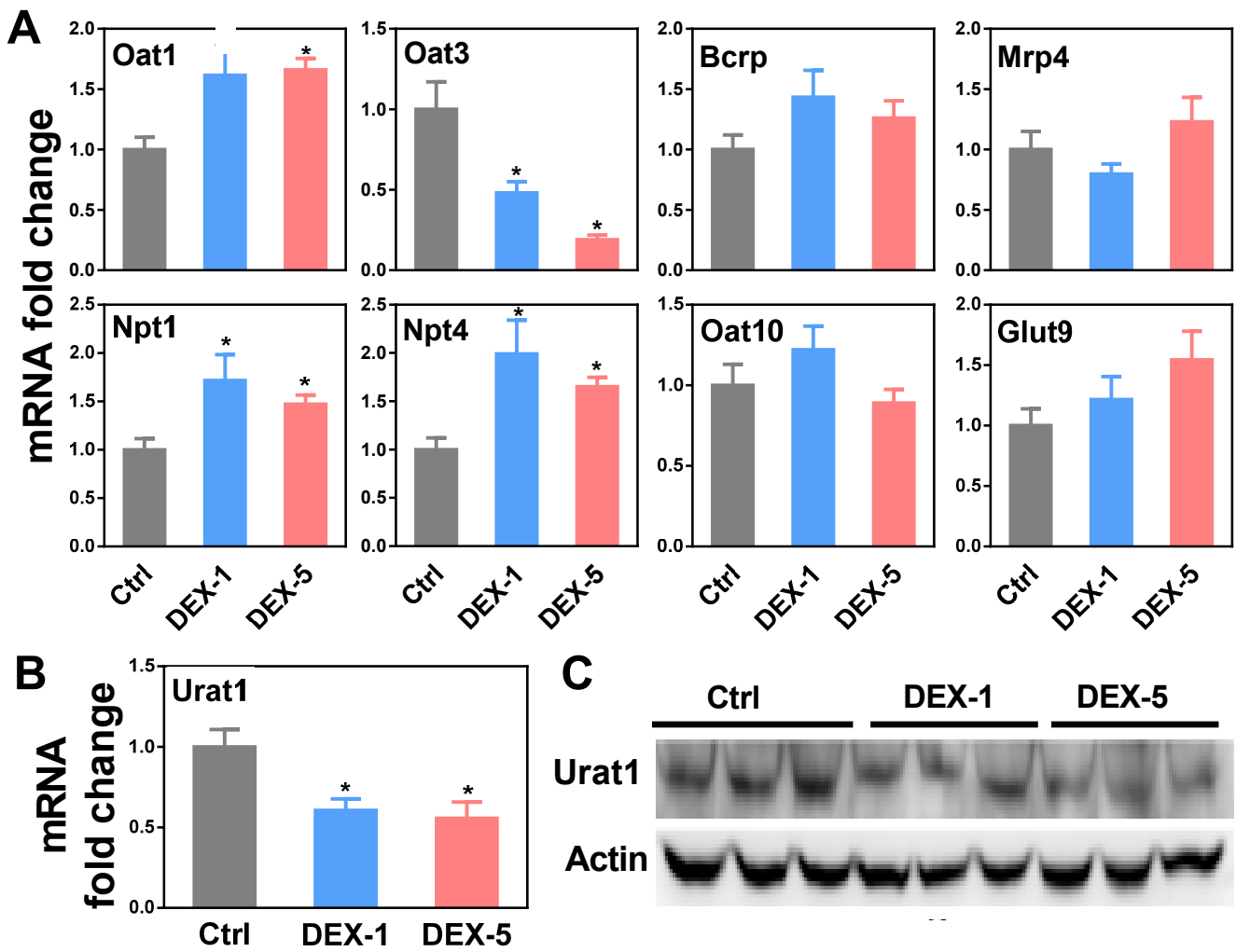


Figure 4

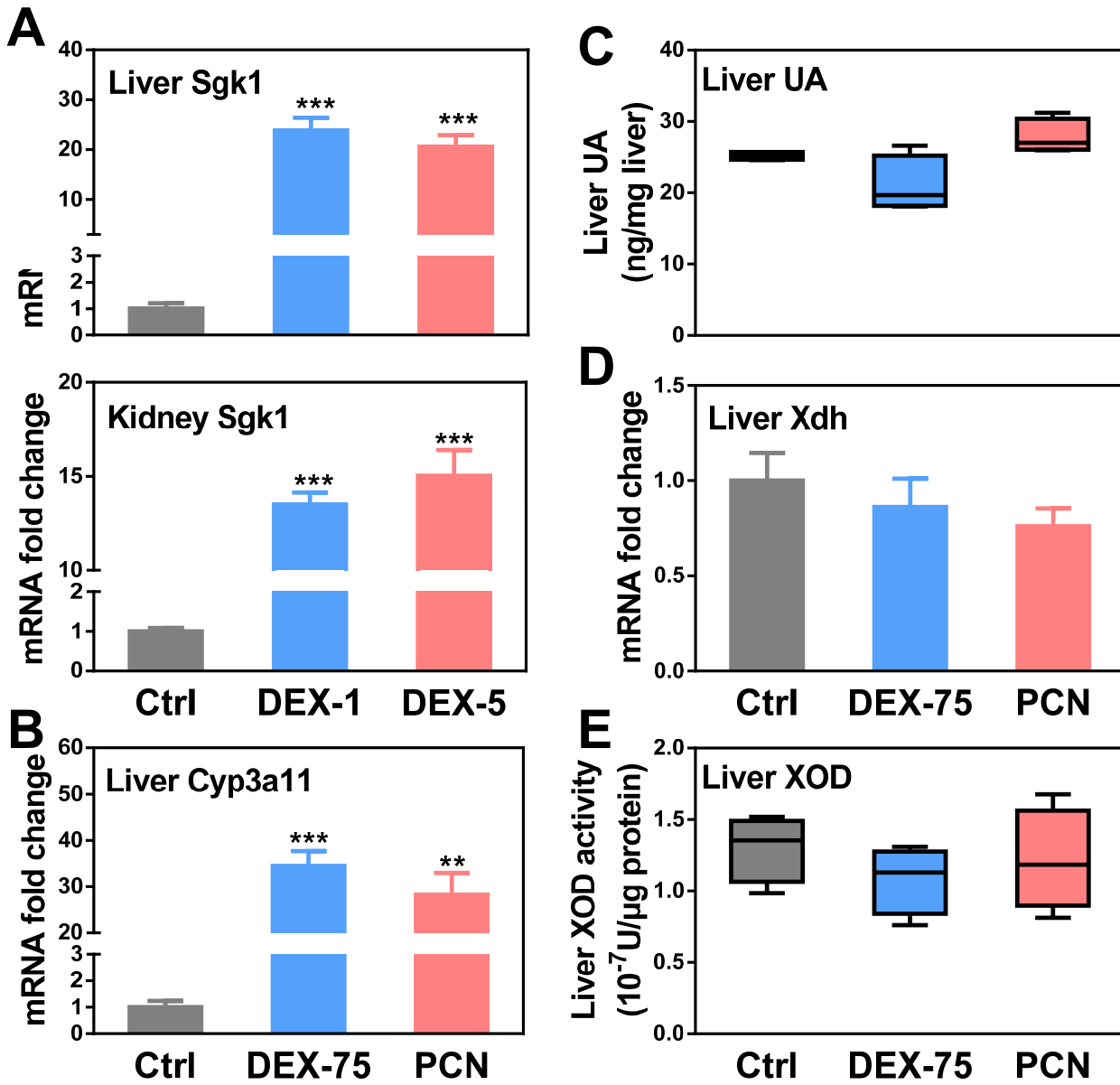


Figure 5

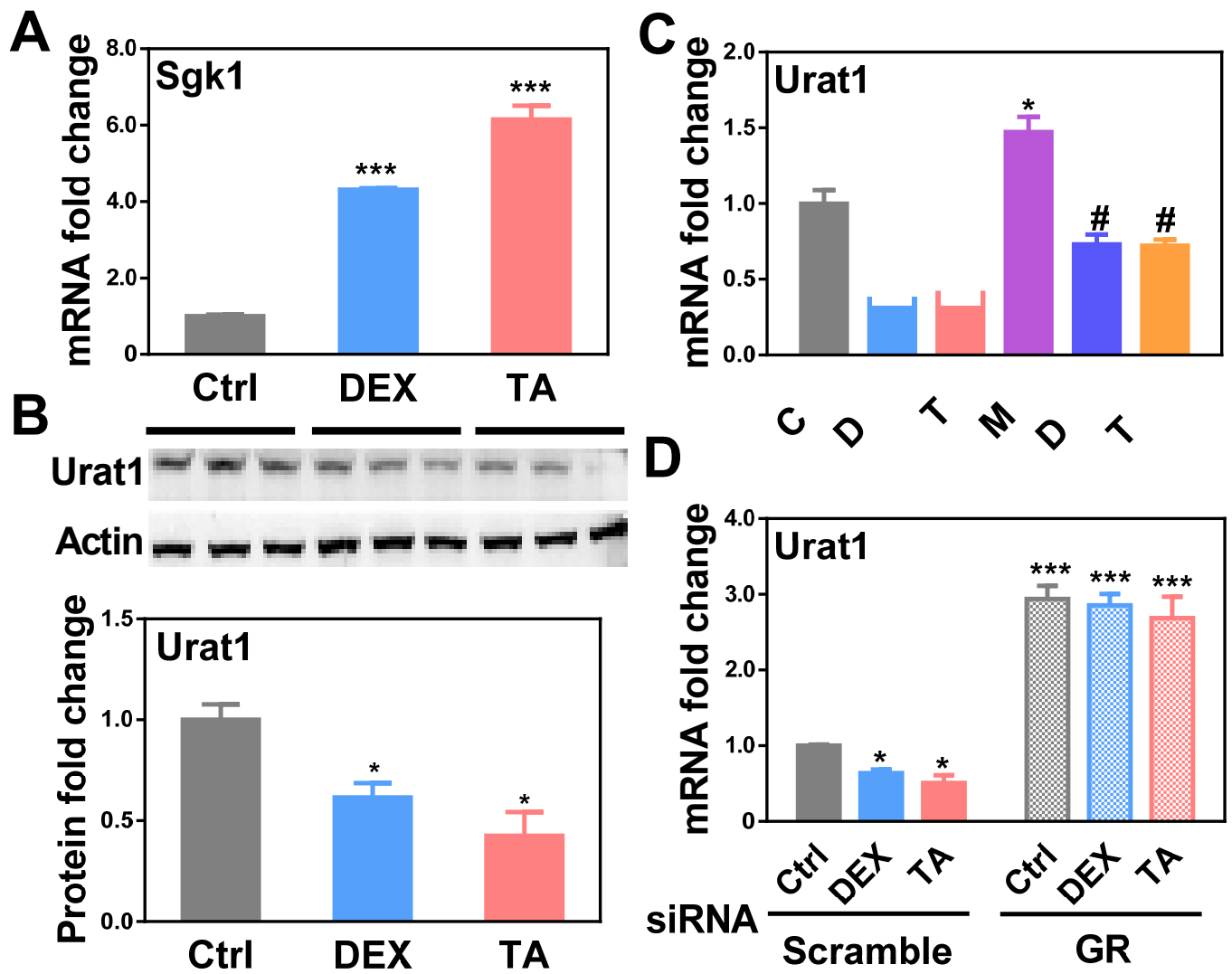


Figure 6

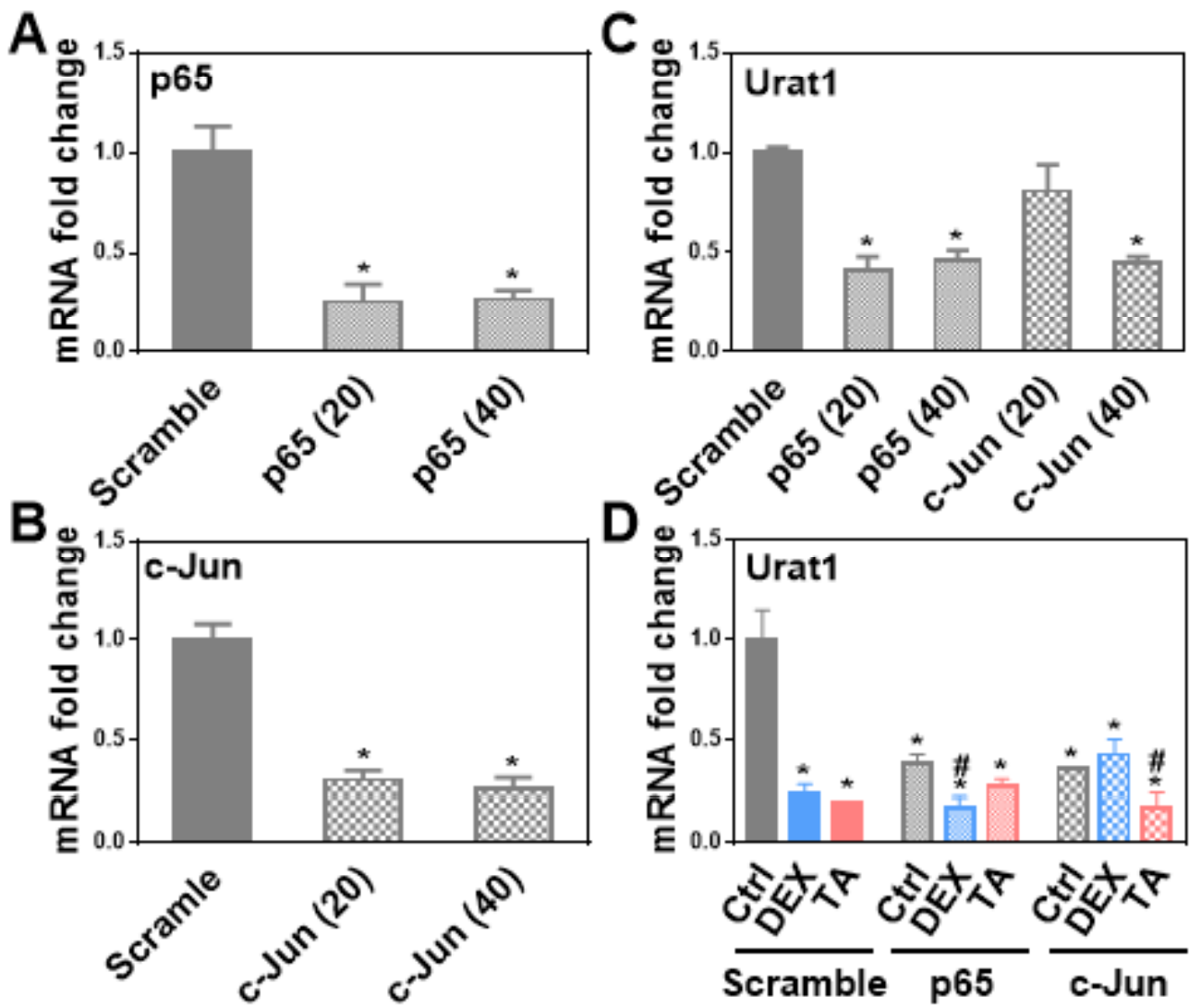
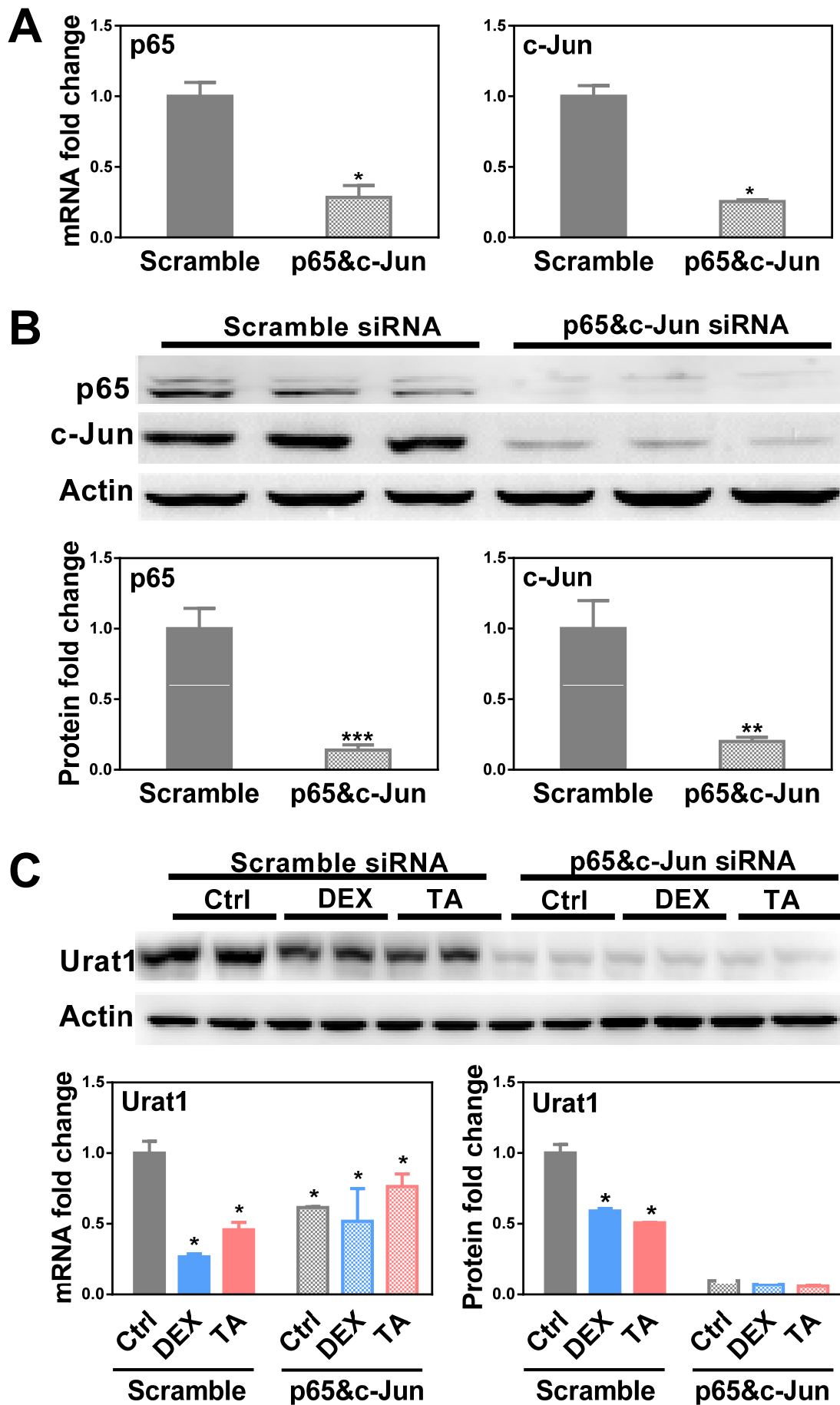


Figure 7



Supplemental Data for:

Glucocorticoids increase renal excretion of urate in mice by down-regulating urate transporter 1

Gentao Li, Lifeng Han, Ruicong Ma, Khawar Saeed, Hui Xiong, Curtis D. Klaassen, Yuanfu Lu and Youcai Zhang*

Drug Metabolism and Disposition

Supplemental Table 1. Sequences of the gene-specific siRNA used in this study

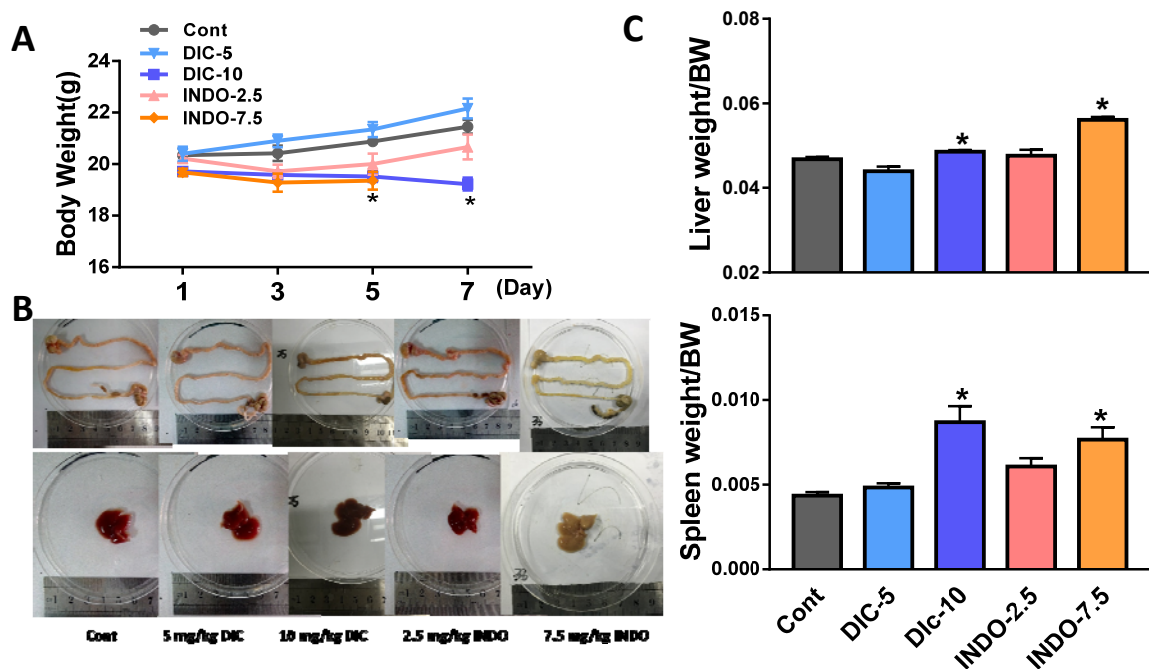
Gene name	Sense sequence (5'→3')	Antisense sequence (5'→3')
Gr	UUACCUUUGUGCUGGAAGAdTdT	UCUUCCAGCACAAAGGUAAAdTdT
P65	GAAGCAUUAACUUCCCUGAdTdT	UCAGGGAAGUUA AUGCUUCdTdT
c-Jun	GGCACAGCUUAAACAGAAAdTdT	UUUCUGUUUAAGCUGUGCCdTdT

Supplemental Table 2. PCR primers pairs used for qRT-PCR analysis

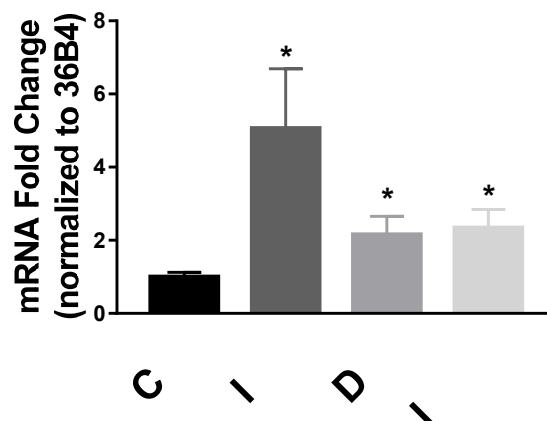
Species	Gene name	Sense primer (5'→3')	Antisense primer (5'→3')
Rat	Urat1	TGCCACCTTCCCCCTCTACTG	GCCCAAAGCTGAAGCCCAAGG
	P65	ACGATCTGTTTCCCCTCATCT	TGCTTCTCTCCCAGGAATA
	C-Jun	CGCGGGAGCCAACCAACGTG	CGTCCCCGCTTCAGTAACAAAGT
	Sgk1	AGAGGCTGGGTGCCAAGGAT	CACTGGGCCCGCTCACATTT
	Gr	GCTCCACCTTGTCTCCA	TCCAGTTTTTCAGAACCAACAGG
	36b4	GGCATCACCCTAAAATCTCC	GCTCCACCTTGTCTCCA
Mouse	Urat1	CGTGGGACCTGGTATGTAAC	CCAAACCTATCTGAGGCATGG
	Sgk1	CTGCTCGAAGCACCCCTTACC	TCCTGAGGATGGGACATTTTCA
	Tat	TCTGGAGCCATGTACCTTATGG	TTCCACGTCATTCTCAAATTCTG
	Ppat	TGCCAGCCATTTCGTAGTTGAG	AGGACGTGGAAAGCCCAATAC
	Prps	ATGCCTAACATCGTGCTCTTC	GATCTCGACACTGGTCTCCTG
	Ada	GTCACCCCTGATGACGTTGTG	CAGAATGGACCGGACCTTGAT
	Hprt	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
	Xdh	ATGACCGCCTTCAGAACAAGA	TGTCACAGCAACATGATGCAA
	Uricase	GAAGTGGAAATTTGTCCGAAC	TGG
	Cyp3a11	ACAAACAAGCAGGGATGGAC	GGTAGAGGAGCACCAAGCTG
	Glut9	CTTCCTCTACGGGTACAACCT	CAGAGCAGAGTCAGGGTATCC
	Npt1	CCAAGGACCACCCGTATATGA	CTTTGATTGGCAGGGATTGTCT
	Npt4	CATGAAGCCTCAAAGCATCTTCC	TTCCCTGCCAAGTATCCTCCA
	Bcrp	CTGGCCTTAATGCTATTCTGGG	TTGAAATGGGCAGGTTGAGGT
	Mrp4	GGCACTCCGGTTAAGTAACTC	TGTCACCTTGGTCGAATTTGTTCA
	Oat1	AATGGCACCGGAGTCCAAG	AAGGTTCCACTCAGTCACGAT
	Oat3	ACCGAGCCATGCTTGGATG	GCCAGCAAGAGATAGCTCCAG
	36b4	GGACCCGAGAAGACCTCCTT	CCAACAGCATATCCCGAATC

Supplementary Figures

Supplemental Figure 1. The effects of DIC and INDO on body weights (A), the morphology of livers and intestine (B), as well as the ratio of liver and spleen weights to body weights (C) in mice after 7-day oral administration. Mice were orally administered with DIC (5 and 10 mg/kg) and INDO (2.5 and 7.5 mg/kg) daily for 7 days. The data are expressed as mean \pm SEM, N=5. * $p < 0.05$ versus control group.



Supplemental Figure 2. The mRNA expression of NAG-1 in livers of mice. The mice were orally administered with 30 mg/kg IBU, 5 mg/kg DIC, and 2.5 mg/kg INDO daily for 7 days. The mRNA expression of NAG-1 was quantified using RT-PCR. The relative mRNA levels were calculated as fold change normalize to controls. The data are expressed as mean \pm SEM, N=5. * $p < 0.05$ versus control group.



Supplemental Figure 3. Male C57/BL6 mice (N=5/group) were administered DEX (1 and 5 mg/kg, i.p.) for 7 days. The mRNA levels of Ppat, Prps, Hpvt, Ada, and Uricase in livers of mice were quantified using RT-PCR. Data are expressed as mean \pm SEM (N=5). *P < 0.05 versus control group.

