Uric acid upregulates the adiponectin-adiponectin receptor 1 pathway in renal proximal tubule epithelial cells

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Abstract. Adiponectin (APN) is a protein hormone that is primarily derived from adipocytes. It can also be secreted by renal cells. Hypoadiponectinemia has been documented in patients with hyperuricemia, however, whether soluble uric acid (SUA) regulates the expression of APN and APN receptor 1 (AdipoR1) in renal proximal tubule epithelial cells (PTECs) remains to be elucidated. The present study investigated the expression of APN and AdipoR1 in cultured PTECs that were exposed to SUA through immunofluorescence and western blot analysis. In addition, Sprague-Dawley rats with oxonic acid-induced hyperuricemia (HUA) with or without febuxostat treatment were employed as an animal model to measure 24 h urine protein, serum creatinine, urea nitrogen, uric acid and homeostasis model assessment of insulin resistance. Renal pathology was evaluated using hematoxylin and eosin and immunohistochemical staining. APN and AdipoR1 expression in the renal cortex were evaluated by western blotting. The results demonstrated that, in PTECs, the expression of APN and AdipoR1 was constant and increased upon SUA exposure. Similar observations were made within the proximal renal tubules of rats, and the oxonic acid-induced increases in APN and AdipoR1 were offset by febuxostat treatment. Furthermore, SUA-treated PTECs exhibited an increase in the expression of NLR family pyrin domain-containing (NLRP) 3, which was dose-dependent. NLRP3 expression was also significantly increased in the renal cortex of HUA rats compared with control and febuxostat-treated rats. In conclusion, SUA enhanced the expression of APN and AdipoR1 in PTECs, which was associated with an increase in NLRP3

expression. The APN-AdipoR1 pathway was demonstrated to have an important role in *in vitro* and *in vivo* models of renal proximal tubule inflammatory injury. Therefore, this pathway may be a potential therapy target in urate nephropathy.

Introduction

Adiponectin (APN), a 30-kDa protein hormone, is primarily secreted by adipocytes into the circulation and is encoded by apM1. It is also secreted by the kidney (1-3) and placenta (4). The bioactivity of APN is mediated through APN receptors (AdipoRs), among which AdipoR1 and AdipoR2 are well defined and are expressed at different locations with different functions (5). AdipoR1 binds to globular APN (gAd) to activate and phosphorylate adenosine monophosphate-activated protein kinase (AMPK) in the kidney. AdipoR1 action is similar in the skeletal muscle, synovial fibroblasts, atrial cells and endothelial cells (1,5-10). AdipoR2 is primarily expressed in the liver and weakly expressed in the kidney, and mediates the activation of peroxisome proliferator-activated receptor α (5,11-13). Studies have demonstrated that AdipoR1 protects the kidney against inflammatory, fibrotic or oxidative damage through the activation of AMPK (14-19).

The role of circulating APN has been widely investigated in different nephropathies. In patients with type 2 diabetes, circulating APN is reduced in the absence of diabetic nephropathy or during early nephropathy (20,21), but is increased when advanced albuminuria develops (21). In adenine-induced chronic kidney disease models, APN levels in the serum and urine are increased (22). In the offspring of rats receiving a high-fat and high-fructose diet, hypoadiponectinemia was observed, which was accompanied by increased urinary albumin excretion (UAE), glomerulosclerosis and renal transforming growth factor-β1 expression, and reduced podocin (19). In addition, serum APN levels are reported to be inversely correlated with UAE (19). These results indicate that circulating APN may protect against renal injury. Hypoadiponectinemia is associated with a lower degree of renoprotection and is therefore associated with albuminuria during early diabetes. Higher serum APN levels during established diabetic nephropathy may be a protective response to mitigate renal lesions.

However, blood may not be an optimal choice for measuring APN levels, as serum APN is influenced by a

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low glomerular filtration rate or by medication (14). There is limited evidence concerning the intrarenal distribution of APN in kidney diseases. The expression of APN in glomerular endothelia is dramatically reduced in patients with diabetic nephropathy (2) or systemic lupus erythematosus with glomerular hypercellularity, sclerosis and interstitial inflammation (3). In an adenine-induced chronic kidney disease model, renal AdipoR1 and AdipoR2 mRNA was elevated and positively correlated with serum and urinary APN levels (22). These findings indicate that the local APN pathway may also be protective against renal injury. However, Perri *et al* (11) reported an increase in APN protein, AdipoR1 mRNA and the downstream phosphorylated (p)-AMPK/p-extracellular signal-regulated kinase/p-c-Jun N-terminal kinase (JNK) activities in lipopolysaccharide (LPS)-treated HK-2 cells. Notably, APN mediates the nuclear translocation of nuclear factor-κB (NF-κB) and p-c-Fos/p-c-Jun (activator protein 1), which are both induced by pJNK and promote the transactivation of tumor necrosis factor (TNF)-α, thereby worsening renal inflammation in an autocrine-dependent manner instead of exerting an anti-inflammatory effect (11). This controversial finding challenges the physiological effect of APN and merits further investigation.

Uric acid is an independent predictor of the development, progression and prognosis of chronic kidney disease (23-27). It causes renal inflammation in a crystal-dependent and independent manner (28). Soluble uric acid (SUA) has been reported to induce inflammation through neutrophil and monocyte chemotaxis (29,30) or through the activation of an NLR family pyrin domain-containing (NLRP) 3 inflammasome and the secretion of TNF-α (31). Our previous study demonstrated that SUA activated NLRP3 and increased interleukin (IL) 1β production in cultured primary human renal proximal tubule epithelial cells (PTECs) (32). Based on the association between inflammation and APN signaling, APN may have a critical role in SUA-induced inflammation. However, to the best of our knowledge, no data currently exists to support or refute this hypothesis.

In the current study, we evaluated the *in vivo* intrarenal expression of APN and AdipoR1 by immunohistochemistry and the renal pathology in a hyperuricemic (HUA) model. We further investigated the protein expressions of APN pathway and that of NLRP3 by immunoblotting and immunofluorescence in cultured PTECs exposed to SUA.

Materials and methods

Materials. The primary human renal PTEC cell line (cat. no. 4100) and epithelial cell culture medium (EpiCM; cat. no. 4101) were provided by ScienCell Research Laboratories, Inc. (San Diego, CA, USA). BioXtra uric acid and oxonic acid potassium salt were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Primary antibodies against APN and AdipoR1 were from Abcam (Cambridge, UK), and anti-NLRP3 was purchased from Novus Biologicals, LLC (Littleton, CO, USA). GAPDH primary antibody and goat anti-mouse and goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibodies for immunoblotting were obtained from Sino Biological, Inc. (Beijing, China). For immunohistochemistry, the goat anti-mouse and goat

anti-rabbit fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were from Abcam (Cambridge, UK). The goat anti-mouse and goat anti-rabbit IgG HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Hydrogen peroxide (cat. no. ZLI-9312), ethylenediaminetetraacetic acid (EDTA; cat. no. ZLI-9068) solution for antigen retrieval and bovine serum albumin (BSA; cat. no. ZLI-9027) were purchased from OriGene Technologies, Inc. (Beijing, China). 3,3'-diaminobenzidine (DAB) and DAPI were provided by OriGene Technologies, Inc. Instant hematoxylin and eosin (H&E; cat. nos. ar1180-1 and ar1180-2) solution were purchased from Boster Biological Technology (Pleasanton, CA, USA).

SUA preparation. SUA was freshly prepared prior to each experiment as previously described (33). Briefly, the BioXtra uric acid was dissolved in 1 M NaOH at a final concentration of 50 mg/ml. The solution was filtered (22 µm pore size) and tested for mycoplasma and endotoxin (31). There were no detectable crystals (polarizing microscopy) in the solution neither at the onset nor during the incubation period.

Cell culture. PTECs were incubated at 37°C in a humidified 5% CO_2 and 95% air atmosphere. Cells of less than four passages were used in the following experiments. In all experiments, PTECs were cultured in epithelial cell medium, which comprised 500 ml basal medium, 50 ml fetal bovine serum, 5 ml epithelial cell growth supplement and 5 ml penicillin/streptomycin solution (32). The medium was changed every other day.

Cell viability. Growth-arrested PTECs were seeded onto 96-well plates ($0.25x10^5$ cells per well) and exposed to SUA at increasing concentrations (0, 12.5, 25, 50, 100, 150 and 200 µg/ml) for different durations (24, 48 and 72 h). Control cells were treated with blank medium. A commercial MTT assay kit (Amresco, LLC, Solon, OH, USA) was used to measure the cell viability of PTECs. Cells were incubated with MTT solution for 4 h and crystals were dissolved in dimethyl sulfoxide. Cell viability was recorded as a percentage change in the absorbance (570 nm) of treated cells compared with the control cells.

HUA rat models. Male, 8-weeks-old specific pathogen-free Sprague-Dawley rats (n=12; 200-250 g) were bred at the Animal Center in the School of Pharmacy, Fudan University (Shanghai, China). Rats were kept in groups in a 12 h light/dark cycle at 25°C with free access to food and water. Rats were acclimatized for 1 week prior to being divided into the following three groups (n=4 per group): Control group, where mice received routine chow; HUA group, which received oral administration of a uricase inhibitor, oxonic acid (750 mg/kg), daily for 10 weeks; and the febuxostat (FEB) group, which received 10 weeks oral gavage of oxonic acid (750 mg/kg) daily, with 4 weeks gastric FEB (5 mg/kg) daily after 6 weeks of oxonic acid treatment. After 10 weeks, rats were intraperitoneally anesthetized with 2% sodium pentobarbital (40 mg/kg). Blood was collected into non-heparinized tubes by cardiac puncture after 8 h fasting until rats died due to exsanguination. Blood was centrifuged at 2,000 x g for 10 min at 4°C to harvest

sera samples. Serum creatinine (SCr), uric acid, blood urea nitrogen (BUN) and glucose levels were determined using a P800 automatic biochemical analyzer (Roche Diagnostics Beijing, China). Fasting insulin (FINS) was measured with a i2000 chemiluminescence analyzer (Abbott Pharmaceutical Co., Ltd., Lake Bluff, IL, USA). Homeostasis model assessment-insulin resistance (HOMA-IR) was also deduced, as described previously (34). Urine samples were collected 24 h prior to sacrifice. The 24 h urinary protein (UP) was detected with the sulfosalicylic acid method using a BN ProSpec® protein analyzer (Siemens Healthineers, Erlangen, Germany). Kidneys were collected and weighed, and the renal cortex was fixed with 4% paraformaldehyde for 24 h at room temperature, embedded in paraffin and submitted for H&E and immunohistochemical staining. The remaining tissues were stored at -80°C for western blotting assays. All animal procedures were in accordance with National Institute of Health guidelines (35) and were approved by the Animal Care and Use Committee of Fudan University (Shanghai, China).

Immunofluorescence, histological and immunohistochemical staining. Following treatment with control or 100 µg/ml SUA for 48 h, PTECs were seeded at a density of $4x10^5$ cells/well and were fixed with 4% paraformaldehyde pre-cooled at 4°C for 15 min, permeabilized with 0.2% Triton-X-100 for 15 min and blocked with 10% BSA (OriGene Technologies, Inc.) for 1 h at room temperature, prior to incubation with antibodies against APN (cat. no. ab22554; 1:100) and AdipoR1 (cat. no. ab126611; 1:200) for 1 h at room temperature. All dilutions were made in 1% BSA in PBS. Subsequently, cells were washed three times with PBS prior to incubation with goat anti-mouse (cat. no. ab6785; 1:2,000) and anti-rabbit (cat. no. ab6717; 1:500) FITC-conjugated secondary antibodies for 50 min at 37°C, followed by PBS washing and staining with DAPI for 5 min at room temperature. Images were acquired using a confocal fluorescence microscope (Leica TCS-SP5; Leica Microsystems GmbH, Wetzlar, Germany). Two pathologists evaluated the cellular localization of each protein in a blinded manner.

In rats, renal tissue samples were fixed in 4% paraformaldehyde at room temperature for 24 h, dehydrated in a graded alcohol series, washed in xylene and paraffin-embedded prior to being cut into 3 µm sections. Instant H&E solution was subsequently used to stain renal slides for 6 min at room temperature. Intrarenal APN, AdipoR1 and NLRP3 expression was determined by immunohistochemistry and semi-quantification on 3 µm paraffin-embedded sections of renal cortices (2). Sections were deparaffinized, endogenous peroxidase activity was blocked with 3% hydrogen peroxide (OriGene Technologies, Inc.) and antigens were retrieved by EDTA (cat. no. ZLI-9068; 1:50) in a microwave oven for 20 min. Slides were blocked in 5% BSA (OriGene Technologies, Inc.) at 37°C for 30 min. Primary antibodies against APN (cat. no. Ab22554, 1:1,000), AdipoR1 (cat. no. Ab 126611, 1:2,000) and NLRP3 (cat. no. NBP2-12446, 1:1,000) were subsequently incubated at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (cat. no. sc-2012; 1:200 and cat. no. sc-2005; 1:200) purchased from Santa Cruz Biotechnology, Inc. at 37°C for 1 h. The color reaction was induced by DAB, followed

by counterstaining with 10% Mayer's hematoxylin at room temperature for 1 min. PBS was used as the negative control. Two pathologists blinded to the nature of samples reviewed histological sections under light microscopy. Positive signals were indicated as brown. Quantification was performed by examining 10 (magnification, x400) fields per sample. The area percentage for the stained tubules was evaluated by eye and the proportion score (PS) was graded as 0 (no positive staining), 1 (≤5%), 2 (6-10%), 3 (11-20%) or 4 (>20%). The intensity of staining was assessed as intensity score (IS) and was scaled as 0 (no staining), 1 (weak staining or light yellow), 2 (moderate staining or yellowish brown) or 3 (strong staining or brown). The final histological score was expressed as a product of PS and IS, as described by Yu *et al* (36).

Western blot analysis. Upon confluence, PTECs were exposed to freshly prepared SUA solution (0, 50, 100 and 200 µg/ml) in basal medium for 48 h. Cells were subsequently harvested in cold PBS and suspended in total radioimmunoprecipitation assay buffer (RIPA; Beyotime Institute of Biotechnology, Haimen, China). Rats renal cortex tissue was collected and lysed in RIPA buffer (Sigma-Aldrich; Merck KGaA). The concentrations of total protein in cell lysates were determined by a Bio-Rad Protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the Bradford method. Lysates (40 µg) were resolved on 11% SDS-PAGE gels, transferred onto a nitrocellulose membrane and probed with antibodies against APN (cat. no. ab22554; 1:1,000), AdipoR1 (cat. no. ab126611, 1:2,000) and NLRP3 (cat. no. NBP2-12446, 1:1,000) at 4°C overnight after 30 min blocking with 5% BSA at room temperature. Membranes were incubated with secondary antibodies, including horseradish peroxidase-conjugated goat anti-mouse (SSA007, 1:1,000) and goat anti-rabbit IgG (SSA004, 1:1,000) antibodies for 1 h at room temperature. Protein immunoblots were developed using RapidStep™ ECL Reagent (cat. no. 345818; Merck KGaA) and analyzed through the ImageQuant LAS 4000 software (GE Healthcare, Chicago, IL, USA). A GAPDH antibody (cat. no. 10094-T52; 1:10,000; Sino Biological, Inc.) was used as an internal control.

Statistical analysis. All statistical analyses were performed using SPSS 21.0 software (IBM Corp., Armonk, NY, USA). Normally distributed data, including kidney/body mass ratio, BUN, SCr, serum uric acid, HOMA-IR and 24 h urinary protein (UP) for rats, the cell viability of PTECs, the expression levels of APN, AdipoR1 and NLRP3 protein in cultured PTECs or renal tissues from rats were expressed as the mean ± standard deviation. Discrepancies between groups were analyzed by one-way analysis of variance and Least Significant Difference test. Non-normally distributed data such as the renal histological scores of rats in different groups were analyzed by Kruskal-Wallis and Nemenyi test. P<0.05 was considered to indicate a statistically significant difference.

Results

Viability of PTECs exposed to SUA. PTECs seeded in 96-well plates were incubated with different concentrations of SUA (12.5-200 µg/ml) for predefined periods. Cell viabilities were assessed by a commercial MTT assay. As demonstrated in

Fig. 1, increasing concentrations of SUA had no significant effect on the cell viability of PTECs compared with the blank medium treatment. As 100 μg/ml uric acid is a common hyperuricemia level in human serum, this dose was employed in the following experiments.

Biochemical alterations in HUA rat models. All rats survived the treatment with no alterations in their eating and drinking behavior. The behavior of rats was evaluated through observing the appetite of each animal. Following sacrifice, only serum uric acid levels exhibited significant differences between the groups, and no obvious differences were observed for kidney/body mass ratio, BUN, SCr, 24 h UP or the HOMA-IR (Table I).

Immunofluorescence analysis of the expression of APN and AdipoR1 in control and SUA-treated PTECs. The control PTECs cultured in basal medium exhibited positive staining for APN and AdipoR1 within the cytoplasm (Fig. 2A). Compared with the basal medium control cells, SUA (100 μg/ml) incubation for 48 h markedly increased the expression of cytoplasmic APN and transmembrane AdipoR1 in cultured PTECs (Fig. 2A).

SUA promotes the expression of APN signaling in PTECs and HUA rats. Western blotting demonstrated that APN (Fig. 2B and C) and AdipoR1 (Fig. 2B and D) protein levels in PTEC cell lysates were significantly increased dose-dependently compared with control cells following treatment with 50 and 100 μg/ml SUA, with APN and AdiopoR1 levels peaking at 100 μg/ml SUA. However, expression levels declined following treatment with 200 μg/ml SUA (Fig. 2B-D).

There was visible *in vivo* APN and AdipoR1 immunohistochemical staining within proximal renal tubules of control rats. APN localized predominantly in the cytoplasm of the renal tubular epithelia, whereas AdipoR1 was detectable on the membrane and within the cytoplasm (Fig. 3A). Immunohistochemistry staining revealed intense staining of APN and AdipoR1 within the proximal renal tubules in HUA rats compared with the control group, however, staining was weaker in the FEB group (Fig. 3A). Tubular APN and AdipoR1 were quantitatively higher in HUA rats compared with the control group, while FEB treatment significantly reduced the expression of APN and AdipoR1 in HUA rats (Fig. 3B). Furthermore, western blot analysis also demonstrated that APN levels were markedly higher in HUA rats compared with the control group, while APN levels in the FEB group were significantly lower compared with the HUA group (Fig. 3C and D). No statistically significant difference was observed in AdipoR1 expression between the HUA and control groups, however, AdipoR1 levels were significantly lower in the FEB group compared with the HUA group (Fig. 3C and D).

SUA induces renal inflammation. HUA rats exhibited a prominent loss of brush borders, detachment of renal tubular epithelial cells and interstitial mononuclear cell infiltration, compared with the control and FEB groups (Fig. 4A). In addition, intense NLRP3 staining within the tubules of HUA rats, and not control or FEB groups, was observed (Fig. 4B). In

addition, protein levels of NLRP3 in the renal cortices were significantly higher in HUA rats compared with the control and FEB groups (Fig. 4C). Furthermore, PTECs exposed to SUA for 48 h exhibited increased NLRP3 expression in a dose-dependent manner (Fig. 4D). NLRP3 levels declined following treatment with an SUA concentration of 200 μg/ml (Fig. 4D).

Discussion

Our previous study demonstrated that SUA is a newly-recognized danger signal and induces the activation of the NLRP3 inflammasome, maturation of caspase-1, cleavage of IL-1β and the overexpression of intercellular cell adhesion molecule (ICAM)-1 through toll-like receptor (TLR)4 in human PTECs (32). However, it is unclear whether SUA, as a trigger of inflammation, stimulates the expression of APN and its receptor. In the present study, the results demonstrated that APN and its receptor AdipoR1 were constantly expressed in human PTECs, and were simultaneously upregulated upon exposure to SUA, which was also associated with increased NLRP3 expression. These findings indicate that the APN-AdipoR1 axis may participate in the modulation of SUA-induced inflammatory reactions in human PTECs.

It is established that APN is an adipocyte-derived cytokine (37) that exhibits extensive effects, including insulin-sensitization, anti-inflammatory, anti-fibrotic and anti-atherosclerosis effects, and lowers the risk of metabolic disorders (6,12,38-40). Certain studies have focused on APN signaling in the kidneys (1,3,11) and have demonstrated that APN was detectable on the endothelial surfaces of glomerular capillaries, podocytes, intrarenal arterioles, peritubular capillaries, smooth muscle cells and tubular epithelial cells in healthy and injured kidneys (1-3), in addition to cultured PTECs (11). APN is reported to protect podocytes against oxidative stress (16,17), inhibit glycogen synthase in distal tubules (13), mitigate interlobular arteriosclerosis (41) and prevent renal diseases in the offspring of maternal rats that were exposed to a high-fat and high-fructose diet (19). APN expression was also modulated by metabolic dysfunction in the presence of hypertension or hyperuricemia in chronic kidney disease (42,43). Baldwin *et al* (44) demonstrated that the levels of APN mRNA in fat tissues and circulating APN were reduced in obese mice with metabolic syndrome and hyperuricemia, while levels were increased upon lowering uric acid with allopurinol. In patients with hyperuricemia, circulating APN levels were also reduced (45), and increased serum uric acid levels were associated with hypoadiponectinemia in patients with essential hypertension (46). Therefore, circulating APN is associated with hyperuricemia. Furthermore, serum APN levels may be used to predict the risk of mortality and cardiovascular disease in patients with renal disease in a biphasic manner; a high APN serum level (>20 mg/l) is associated with higher cardiovascular risk, while lower serum APN levels (<15 mg/l) also predict a higher cardiovascular risk (14). However, the effects of systemic APN are not identical to local renal APN, and limited information is available concerning the biological importance of APN in renal cells following SUA administration. Therefore, the present study focused on the role of local renal APN in HUA-induced kidney injury. To the

Table I. Serum and urine analysis of rats in different treatment groups.

Parameter	Control (n=4)	HUA (n=4)	FEB (n=4)
KI	0.62±0.04	0.66±0.04	0.64±0.07
BUN, mmol/l	7.08±0.74	5.95±0.32	6.40±1.16
SCr, μmol/l	40.78±2.21	41.22±5.13	41.90±4.74
Serum uric acid, μmol/;	83.24±35.23	155±36.18[a]	16.50±1.32[a,b]
HOMA-IR	7.09±1.74	6.29±1.83	7.23±1.14
24 h UP, g	12.42±5.82	11.11±3.90	12.58±2.89

[a]$P<0.01$ vs. control; [b]$P<0.001$ vs. HUA. HUA, hyperuricemia; FEB, febuxostat; KI, kidney/body mass ratio; BUN, blood urea nitrogen; SCr, serum creatinine; HOMA-IR, homeostasis model assessment-insulin resistance; UP, urinary protein.

best of our knowledge, the present study is the first to demonstrate that SUA induced the expression of APN in PTECs *in vitro* and that APN was overexpressed even in rats with mild HUA. Furthermore, this increase was reversible at low uric acid concentrations, indicating that APN may be involved in the initiation and the development of SUA-associated tubular injury. Notably, in the current study, the results for intrarenal expression of APN were consistent with previous reports (11,47). However, contradictory findings in diabetic nephropathy are also present (2). As uric acid is a proinflammatory factor and also a component of metabolic syndrome, it is difficult to determine whether the uric acid-induced APN upregulation is mediated though inflammatory signaling or metabolic pathways. Therefore, the biological effect of APN and its mechanism requires further investigation. As APN was upregulated upon SUA stimulation, future studies should aim to clarify the regulatory function of APN on SUA-induced inflammation. To elucidate the potential mechanism, *in vitro* or *in vivo* APN knockout models may improve the understanding of how APN modulates the tubular inflammation induced by SUA.

Previous studies have revealed that APN receptors, including AdipoR1 and AdipoR2, are expressed in human renal proximal tubule cells, and the expression of AdipoR1 mRNA was higher compared with AdipoR2 expression. Although AdipoR1 consists of 7 transmembrane domains, it is structurally and functionally different from G-protein-coupled receptors. AdipoR1 is the most abundant APN receptor in the kidneys, and expressed by PTECs and various glomerular cells (1,12,13,22). AdipoR1 has a high affinity for the globular domain of APN (5) and transmits its downstream signaling through AMPK phosphorylation (48). In the adenine-induced chronic kidney disease model, intrarenal AdipoR1 expression in the glomerular endothelium and tubular epithelial cells was upregulated, while AdipoR1 mRNA was positively correlated with serum and urine APN, and 24 h UP (22). In patients with uremia, the expression of AdipoR1 in the muscle and circulatory levels were increased, which was also associated with elevated catalytic AMPK, lower acetyl-CoA carboxylase phosphorylation and decreased carnitine palmitoyl transferase levels. These findings indicate that uremia leads to the upregulation of AdipoR1 accompanied and APN resistance (49).

The expression of AdipoR1 in different animal models has been previously investigated. Recently, Akita diabetic mice
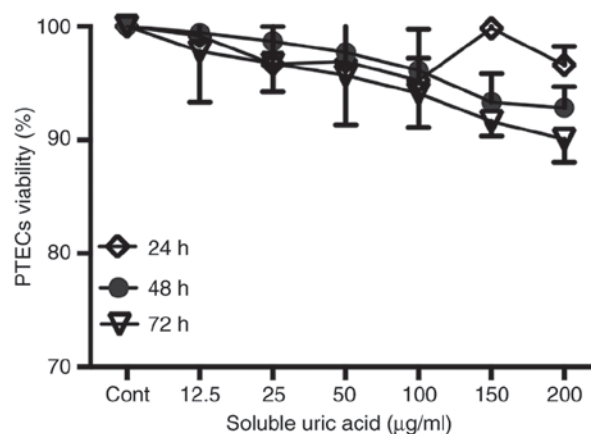

Figure 1. Cell viability of PTECs following SUA exposure. PTECs were incubated with 12.5-200 μg/ml SUA for different durations. As determined by an MTT assay, PTEC viability during different incubation periods did not differ significantly, particularly when the SUA concentration was <100 μg/ml. Results are presented as the mean ± standard deviation of different independent experiment and as a percentage of growth in the control group, which was set at 100%. PTECs, proximal tubule epithelial cells; SUA, soluble uric acid; Cont, control.

with no increases in SCr were reported to exhibit increased glomerular expression of AdipoR1 (50). Inversely, streptozocin (STZ)-induced diabetic rodents (51-53) and mesangial cells (HBYZ-1) exposed to high glucose (53) exhibited AdipoR1 downregulation. However, to the best of our knowledge, the mechanism by which AdipoR1 expression may be regulated in uric acid-treated renal tubule cells has not been previously investigated. In the current study, AdipoR1 was predominantly expressed in renal tubule epithelial cells of rats, with increased levels in rats with oxonic acid-induced hyperuricemia and in cultured human PTECs, compared with the respective control groups. The protein levels of AdipoR1 during SUA exposure were modulated in a similar manner to intrarenal APN levels, which indicates that APN may participate in SUA-induced renal injury through AdipoR1. APN was reported to downregulate the inflammatory cytokine monocyte chemotactic protein (MCP)-1 (12) and attenuate angiotensin II-induced NF-κB activation and fibronectin expression in proximal tubular cells (15), which was dependent on AdipoR1 (12,15). Furthermore, it has been demonstrated that APN exerts anti-inflammatory effects and is therefore renoprotective
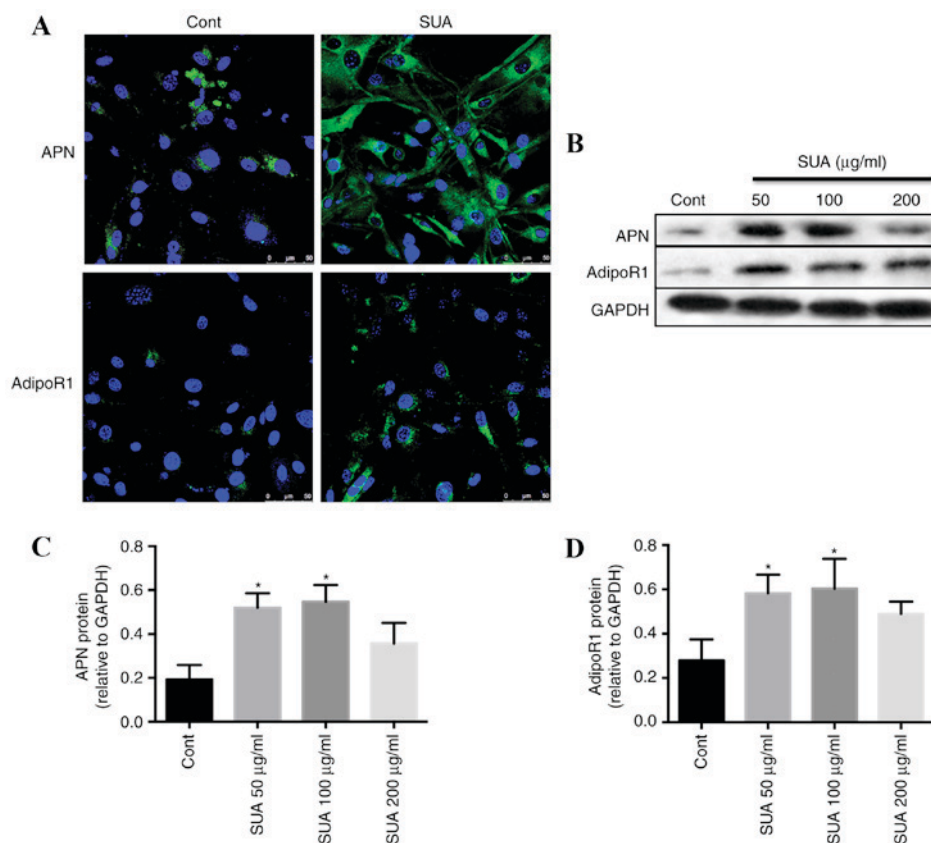
Figure 2. SUA promoted the expression of APN and AdipoR1 in cultured PTECs. (A) APN and AdipoR1 were detected in PTECs cultured in basal medium and exhibited intense staining following incubation with 100 μg/ml SUA for 48 h, as determined by confocal fluorescence microscopy. Magnification, 630x (B) Representative western blot bands for the protein expression of APN and AdipoR1 in PTECs treated with control or 50-200 μg/ml SUA. Quantified protein expression of (C) APN and (D) AdipoR1 in PTECs following different treatments. Protein expression of APN and AdipoR1 in lysates increased compared with the control group following exposure of PTECs to 50 μg/ml SUA and reached a peak at 100 μg/ml SUA. Bar graphs present representative data of four independent experiments. *P<0.05 vs. control group. SUA, soluble uric acid; APN, adiponectin; AdipoR1, APN receptor 1; PTECs, proximal tubule epithelial cells; Cont, control.
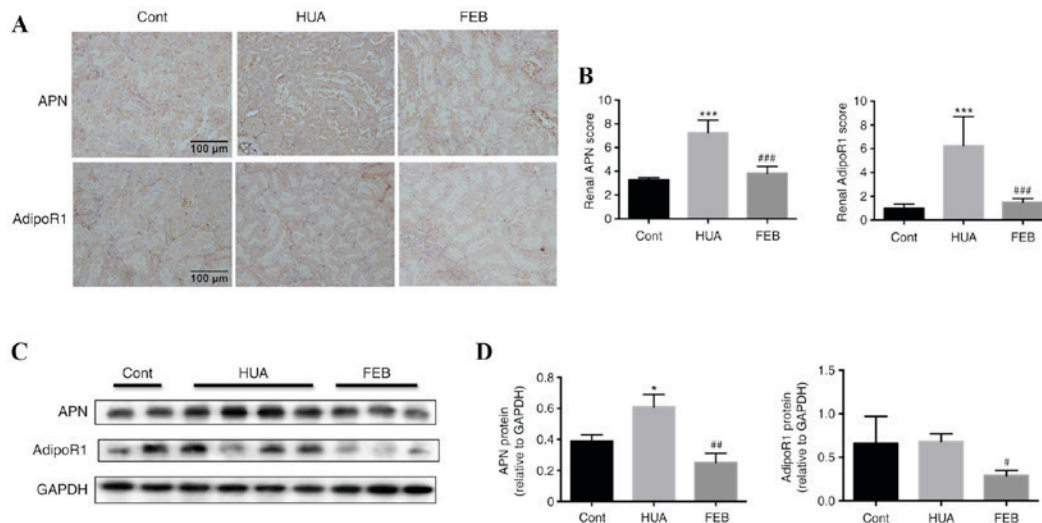

Figure 3. Hyperuricemia enhanced the expression of APN and AdipoR1 *in vivo*. Rats were fed with normal standard chow, oxonic acid or febuxostat for 10 weeks (n=4 per group). (A) Representative images of immunohistochemical staining for intrarenal APN and AdopoR1 in paraffin-embedded renal sections. Magnification, x400. (B) Immunohistochemical staining for APN and AdipoR1 was semi-quantified. (C) Western blotting was performed to investigate the protein expression levels of APN and AdipoR1 in the renal cortex, and representative bands are presented. (D) Densitometric analysis was performed to quantify the protein expression of APN and AdipoR1. *P<0.05 and ***P<0.001 vs. control group; #P<0.05, ##P<0.01 and ###P<0.001 vs. HUA group. APN, adiponectin; AdipoR1, APN receptor 1; HUA, hyperuricemia; Cont, control; FEB, febuxostat.

in various renal diseases. Delaigle *et al* (54) demonstrated that, in human and rodent myotubes, APN was increased *in vivo* and *in vitro* in response to inflammatory stimuli. The upregulation of the APN pathway in renal diseases has also
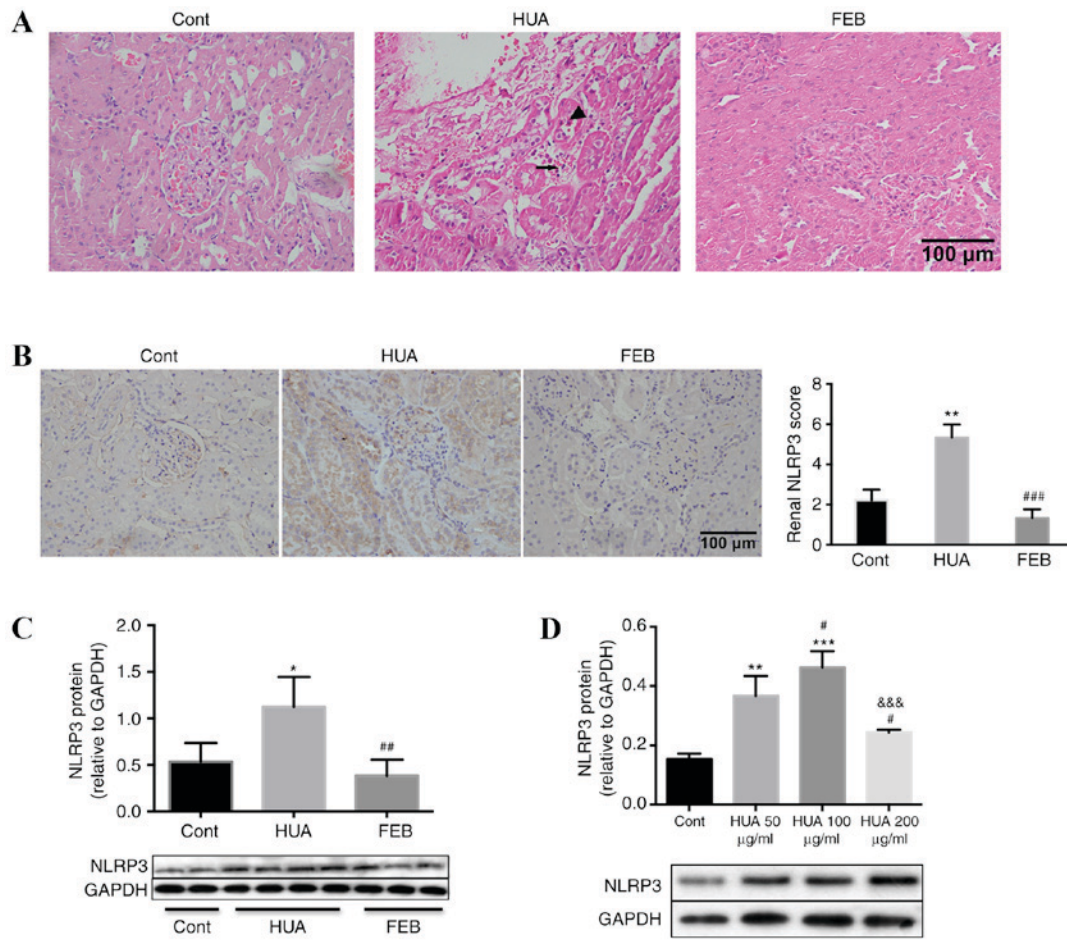
Figure 4. Uric acid induced renal proximal tubule inflammation *in vivo* and *in vitro*. (A) Hematoxylin and eosin staining demonstrated that HUA rats exhibited increased tubular epithelial cell detachment (indicated by arrowhead) and interstitial mononuclear cell infiltration (indicated by arrow) compared with the control and FEB groups. Magnification, x400. (B) Immunohistochemical staining in rat renal tissues also indicated an increased intensity of NLRP3 staining within the tubular cells of HUA rats compared with control and FEB groups, which was evaluated in a blinded manner. Magnification, x400. (C) Western blot analysis demonstrated that NLRP3 protein levels in the renal cortices of HUA rats were significantly higher compared with the control and FEB groups, n=4 per group. (D) Western blot analysis demonstrated that cultured PTECs exhibited a dose-dependent increase in NLRP3 protein levels following treatment with SUA, compared with the control group, although levels were reduced at 200 μg/ml SUA. Bars represent data from four independent experiments. For parts B-D, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. control; for parts B and C, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ vs. HUA group; for part D, $^{\#}P<0.05$ vs. 50 μg/ml SUA group and $^{\&\&\&}P<0.001$ vs. 100 μg/ml SUA group. HUA, hyperuricemia; FEB, febuxostat; NLRP3, NLR family pyrin domain-containing 3; PTECs, proximal tubule epithelial cells; SUA, soluble uric acid; Cont, control.

been considered as compensatory feedback to mitigate renal injuries. Our previous study revealed that SUA activated the NLRP3-caspase-1-IL-1β pathway in cultured PTECs through a TLR4-dependent mechanism (32). In the present study, SUA may have initially activated the NLRP3 inflammasome pathway and subsequently triggered APN-AdipoR1 signaling to mitigate local inflammation. As AdipoR1 is proven to be the most crucial functional receptor for APN, it is reasonable to hypothesize that inflammation and renal damage would be exaggerated if the function of AdipoR1 was inhibited by silencing AdipoR1 in future experiments. Upon silencing of AdipoR1, SUA-induced inflammatory damage would fail to effectively activate the APN-AdipoR1 pathway and subsequently weaken the anti-inflammatory function of APN. Therefore, AdipoR1 knockout studies may clarify the involvement of AdipoR1-mediated APN signaling in the modulation of SUA-induced NLRP3 expression in PTECs.

Previous studies have investigated the role of the APN-AdipoR1 pathway in renal diseases. Evidence regarding the role of APN in renal tissues is controversial. Certain reports indicate that APN is proinflammatory in the kidneys, and the pathophysiology may involve the activation of NF-κB, the recruitment of inflammatory cells, the promotion of macrophage migration and the induction of various cytokines and chemokines, including IL-6, TNF-α, MCP-1 and macrophage inflammatory protein-2 (11,47). In cultured human synovial fibroblasts, recombinant full-length APN was demonstrated to enhance IL-6 expression via the AdipoR1/AMPK/p38/IκB kinaseα/β and NF-κB pathways (7). However, other reports, using overexpression and/or deletion of APN-associated genes and the addition of recombinant APN, have demonstrated that the APN-AdipoR1 pathway possesses anti-inflammatory effects. These effects include the mitigation of macrophage infiltration in the remnant kidney following partial nephrectomy (18), reduced endothelin-1 and plasminogen-1 production in the renal cortex of STZ-induced diabetic rats (55) and the reduction of NF-κB activity in angiotensin II-stimulated human renal tubular cells (15) and high-glucose-treated

mesangial cells (50). The upstream mediators of the inflammatory response were further identified through the use of experimental autoimmune myocarditis models in mice, which indicated that TLR4 may have a critical role in mediating the anti-cardiac inflammation effect of adenovirus-mediated overexpression of APN (56). The results of the current study, in addition to those of previous studies (32,57), indicates potential cross-talk between the NLRP3-IL-1β pathway and APN signaling, in a TLR4-dependent manner. Consistently, in the present study, the renal tissues of HUA rats exhibited marked NLRP3 immunostaining, in addition to structural and inflammatory injuries within the tubulointerstitium. However, no marked damage was observed in the glomerular layer of hyperuricemic rats under light microscopy. These results are consistent with data obtained by Sanchez-Lozada *et al* (58); in oxonic acid-induced rats with mild hyperuricemia, the average serum uric acid concentration was 3.07±0.20 mg/dl and no marked glomerular structural changes were observed. Mazzali *et al* (59) also demonstrated preserved renal architecture in mild hyperuricemia induced by 2% oxonic acid. In the present study, the mean level of serum uric acid in HUA rats was (155.00±36.18) μmol/l, which was lower compared with levels in the study by Sanchez-Lozada *et al* (58). Tubulointerstitial damage was reversed by interventions to reduce uric acid levels in the present study and a previous report (60). The alterations in the tubular APN-AdipoR1 axis upon SUA-induced inflammation indicates that APN-AdipoR1 axis activation may be an autocrine-associated positive feedback mechanism for alleviating renal injuries. Further investigation is required to elucidate the mechanism underlying NLRP3 modulation. To improve the understanding of the SUA-associated alterations in NLRP3 signaling and its association with the APN pathway, the evaluation of serum and renal levels of cytokines downstream of NLRP3, such as IL-18 and IL-1β, may be useful.

APN is a multimeric complex with various molecular weights and isoforms (61), and the phenotype of each isoform may differ (62). Furthermore, recombinant gAd is derived from a wide variety of sources, which may partially explain the contradictory findings concerning APN function in different reports. Standardized procedures regarding the isoform, molecular weight and origin of APN may provide increased information in the future. In addition, the anti-inflammatory properties of APN are beneficial for renal injury and may act against the proinflammatory effects of SUA. To clarify the protective role of APN on SUA-induced inflammation, *in vivo* and *in vitro* APN knockout models are required. To elucidate the mechanism by which the NLRP3 inflammasome interacts with the APN-AdipoR1 signaling pathway, further studies should include AdipoR1 knockout experiments, in addition to AMPK activation or inactivation experiments, to determine whether, and how, the APN-AdipoR1-AMPK pathway regulates the NLRP3 inflammasome in SUA-treated PTECs.

Certain limitations were associated with the present study. For example, the food intake and other behavior indices of rats were not accurately quantified. In addition, the APN and AdipoR1 levels in 200 μg/ml SUA-treated PTECs, unlike those treated with 50 and 100 μg/ml SUA, did not exhibit significant differences compared with the control group. This may be partially attributed to the fact that the effect of SUA on APN, AdipoR1 and NLRP3 expression may not

always be linear, and may also be due to apoptosis occurring in 200 μg/ml SUA-treated PTECs (63). Further study should investigate cells damage prior to changes in cell viability, including necrotic, apoptotic and fibrotic processes of PTECs. Furthermore, an *in vivo* model of renal specific APN-knockout in hyperuricemic animals would improve the understanding of the role of APN in the process of renal tubular inflammation. In summary, the current study demonstrated that SUA regulated the expression of APN and AdipoR1 in PTECs. The APN-AdipoR1 pathway in turn may exert a protective role during SUA-induced renal proximal tubule inflammatory injury and has potential as a viable therapeutic target in urate nephropathy.

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