

Adiponectin protects against uric acid-induced renal tubular epithelial inflammatory responses via the AdipoR1/AMPK signaling pathway

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Abstract. Adiponectin (APN) exerts anti-inflammatory effects in various cells. Uric acid (UA) induces inflammation in proximal renal tubular epithelial cells (PTECs). It remains unknown whether APN protects against UA-induced inflammation. In the present study, human PTECs were incubated with 100 μ g/ml soluble (S) UA in the presence or absence of globular (g) APN, APN receptor 1 (AdipoR1)-short hairpin RNA lentivirus or compound C. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays were performed to assess APN mRNA expression. Immunoblotting was used to assess the protein expression of APN, AdipoR1, NACHT, leucine rich repeat and pyrin domain-containing protein 3 (NLRP3) and the activation of tumor necrosis factor (TNF) α and adenosine monophosphate-activated protein kinase (AMPK). ELISA analyses were performed to assess supernatant levels of interleukin (IL)-1 β and TNF α . It was observed that SUA significantly enhanced APN mRNA and protein expression (both $P < 0.05$) and increased NLRP3 ($P < 0.001$) and TNF α ($P < 0.05$) protein levels, as well as supernatant levels of IL-1 β ($P < 0.01$) and TNF α ($P < 0.001$) compared with untreated cells. gAPN administration significantly limited TNF α synthesis and secretion (both $P < 0.001$), significantly decreased IL-1 β release ($P < 0.01$), impacted NLRP3 protein expression and augmented AdipoR1 protein ($P < 0.01$) and AMPK phosphorylation ($P < 0.05$) levels compared with SUA-treated cells. AdipoR1 knockdown significantly promoted

the synthesis ($P < 0.05$) and release of TNF α ($P < 0.001$), significantly increased IL-1 β supernatant levels ($P < 0.01$) and exhibited little influence on NLRP3 production ($P > 0.05$) compared with the SUA-treated cells. Secreted TNF α levels were significantly increased upon the inhibition of AMPK ($P < 0.05$) and protein levels of IL-1 β , NLRP3 and TNF α in cell lysates were not significantly affected ($P > 0.05$). In summary, the data demonstrated that SUA promoted APN expression in PTECs and that gAPN attenuated SUA-induced inflammation through the AdipoR1/AMPK signaling pathway. AdipoR1 knockdown and AMPK inactivation increased SUA-induced inflammatory damage in PTECs. These findings may help to further understand and regulate UA-associated inflammation in proximal renal tubules.

Introduction

Incidence rates of hyperuricemia have increased during the past decade, with a prevalence of 13.3% in mainland China (1). Hyperuricemia or a high-normal level of serum uric acid (UA) have been demonstrated to be independent risk factors for the initiation and prognosis of chronic kidney disease (2,3), which can be attenuated through urate-reducing therapy (1,4,5). UA, as monosodium crystals or soluble (S) UA, induces tubulointerstitial inflammation (6-8). However, the mechanism by which SUA triggers renal tubular inflammation and how this process is regulated are poorly understood. Serum UA is useful for predicting (9) and screening the incidence of metabolic disorders (10). Additionally, type 2 diabetic patients with hyperuricemia, typically associated with insulin resistance (IR), exhibit an increased incidence of renal calculus compared with patients without hyperuricemia (11). Formation of UA nephrolithiasis decreases the improvement of IR in mice with type 2 diabetes and metabolic syndrome (12). These data suggest that IR may facilitate hyperuricemia-induced development of nephrolithiasis. Thus, the present study evaluates whether IR increases the vulnerability of renal tubules to SUA-elicited inflammation.

Adiponectin (APN) is an adipokine primarily derived from adipocytes (13-15). APN is generally recognized as an

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insulin sensitizer and hypoadiponectinemia caused by genetic and environmental factors impairs insulin sensitivity, leading to diabetes and metabolic syndrome (16). Under conditions of IR, transcription of APN receptor 1 (AdipoR1) (17) and the activation of adenosine monophosphate-activated protein kinase (AMPK) (16) are abolished, decreasing the response sensitivity of APN (16). Over the past two decades, APN has been identified as a potent anti-inflammatory mediator (18-22) via receptor-dependent mechanisms (23). Various studies indicated that APN is expressed in and acts protectively in renal podocytes (18,24,25), mesangial cells (22,26) and tubular epithelial cells (25,27) in humans and rodents (19,28). It was demonstrated that APN knockout (KO) worsens the severity of kidney structural damage, increases the infiltration of macrophages and upregulates the intrarenal production of proinflammatory factors in subtotal nephrectomized and diabetic mice, whereas the overexpression of APN ameliorated these disorders (22,28). Conversely, few studies argued that APN serves as a proinflammatory factor in the renal tubular cell line HK-2 upon stimulation with lipopolysaccharide (LPS) (29) and in acute kidney injury induced by ischemia-reperfusion (30). Therefore, further research is required to clarify the association between APN and renal inflammation. Furthermore, whether and how APN modifies renal tubular inflammation induced by SUA and whether IR-associated abnormal APN signaling facilitates renal tubular injury have not yet been determined.

Here, it was hypothesized that APN conferred resistance in renal tubular inflammation following SUA exposure. Effects of APN and its signaling mechanism in SUA-stimulated human proximal renal tubular epithelial cells (PTECs) with loss-of-function experiment were performed to validate this hypothesis. The present study suggested that APN protects against SUA-induced renal tubular inflammatory responses via the AdipoR1/AMPK signaling pathway.

Materials and methods

Materials and reagents. PTECs (cat. no. 4100) and epithelial cell medium (ECM; cat. no. 4101) were obtained from ScienCell Research Laboratories, Inc. (San Diego, CA, USA). BioXtra UA was purchased from Sigma-Aldrich (cat. no. U0881; Merck KGaA, Darmstadt, Germany). Primary antibodies against APN (cat. no. Ab22554; 1:1,000) AdipoR1 (cat. no. Ab126611; 1:2,000), NACHT, leucine rich repeat and pyrin domain-containing protein 3 (NLRP3; cat. no. Ab109314; 1:1,000) and tumor necrosis factor (TNF) α (cat. no. Ab9635; 1:2,000) were from Abcam (Cambridge, UK). Primary antibodies against AMPK α (cat. no. 2532; 1:1,000) and phosphorylated (p) AMPK α -Thr172 (cat. no. 2535; 1:1,000) were provided by Cell Signaling Technology, Inc. (Danvers, MA, USA). The anti-GAPDH antibody (cat. no. 10094-T52; 1:10,000) and the horseradish peroxidase (HRP)-conjugated goat anti-mouse (cat. no. SSA007; 1:1,000) and anti-rabbit IgG (cat. no. SSA004; 1:1,000) secondary antibodies were from Sino Biological, Inc. (Shanghai, China). High sensitivity ELISA kits for interleukin (IL)-1 β (cat. no. BMS224HS) and TNF- α (cat. no. BMS223HS) were from eBioScience (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The lentivirus-mediated short hairpin (sh) RNA against AdipoR1,

the scramble-shRNA and Polybrene were from Shanghai GeneChem Co., Ltd. (Shanghai, China). Recombinant human globular (g) APN (cat. no. 450-21) was supplied by PeproTech, Inc. (Rocky Hill, NJ, USA). Compound C (cat. no. US1171260), a specific AMPK inhibitor, was from Merck KGaA.

SUA preparation. SUA was freshly prepared prior to each experiment as previously described (31). Briefly, BioXtra UA was dissolved in 1 M NaOH to a final concentration of 50 mg/ml. The solution was filtered (pore size, 0.22 μ m) and tested for mycoplasma and endotoxin. Polarizing microscopy (magnification, x200) was used to check for crystals in the SUA solution for the duration of the experiments.

Cell culture. PTECs were cultured at 37°C in a humidified atmosphere with 5% CO₂ and incubated in ECM, which consisted of basal medium, 2% fetal bovine serum albumin, 1% epithelial cell growth supplement and 1% penicillin/streptomycin solution. Cells passaged 4-7 times were used in the following experiments as previously described (27).

Cell viability. Growth-arrested PTECs were seeded in 96-well plates at 2.5x10⁴ cells/well. Cells were exposed to SUA at increasing concentrations (0, 25, 50, 100 and 200 μ g/ml) for 24, 48 and 72 h. A commercial MTT assay kit from Amresco, LLC (Solon, OH, USA) was used to assess the viability of PTECs following SUA exposure, as previously described (8). In brief, PTECs were incubated with 20 μ l MTT at 37°C for 4 h, followed by an addition of 150 μ l dimethyl sulfoxide. Cell viability is represented as the percentage change in the absorbance measured at 570 nm compared with untreated cells.

Transfection with AdipoR1-shRNA. AdipoR1-shRNA was designed to target the following sequence: 5'-CAAAGC TGAAGAAGAGCAA-3'. The negative control scrambled sequence was: 5'-TTCTCCGAACGTGTCACGT-3'. The uniqueness of these sequences was confirmed using the GenBank/EBI database (<https://www.ncbi.nlm.nih.gov/nucleotide/> and <https://www.ebi.ac.uk/ena>). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot assays were performed to evaluate the efficiency of AdipoR1 silencing. Next, 2x10⁵ PTECs were transfected with 70 μ l AdipoR1-shRNA lentivirus (3x10⁸ transducing U/ml) at a multiplicity of infection = 50 for 24 h prior to incubation for 48 h with 100 μ g/ml SUA. Polybrene (5 μ l/ml) was used to facilitate transfection reactions.

RNA extraction and gene expression levels of APN and AdipoR1. Growth-arrested PTECs (5x10⁵) were exposed to 100 μ g/ml SUA for 4 h. Total RNA was extracted using TRIzol reagent from Takara Bio, Inc. (Otsu, Japan). cDNA synthesis and RT were performed as previously described (8). Briefly, extracted RNA was reverse transcribed into cDNA at 42°C for 50 min and 85°C for 5 min using a PrimeScript™ RT Master Mix kit (Takara Bio, Inc.). qPCR reactions (total volume, 25 μ l) were conducted in duplicate using a SYBR Premix Ex Taq™ kit (Takara Bio, Inc.) and the Fast Real-Time PCR System 7300 (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each reaction was performed at 95°C for 10 min, followed

by 40 cycles of 95°C for 10 sec and 60°C for 30 sec, then 95°C for 15 sec and 60°C for 1 min, and 95°C for 15 sec and 60°C for 15 sec. Primer pairs for amplifying APN (forward, 5'-CTA TGATGGCTCCACTGGTA-3' and reverse, 5'-GAGCATAGC CTTGTCCTTCT-3'; product, 124 bp), *AdipoR1* (forward, 5'-CGGTGGAAGTGGCTGAAGTGA-3' and reverse, 5'-CCG CACCTCCTCTCTTCTT-3'; product, 125 bp) and GAPDH (forward, 5'-ATGGGGAAGGTGAAGGTCTG-3' and reverse, 5'-GGGGTCATTGATGGCAACAATA-3'; product, 107 bp) were provided by Invitrogen (Thermo Fisher Scientific, Inc.). The relative levels of the target mRNAs were normalized and expressed using the $2^{-\Delta\Delta C_q}$ method as described (8,32).

Protein expression levels in cell lysates. PTECs were lysed with lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). Protein concentrations were determined by bicinchoninic acid protein assays (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Immunoblot analyses were conducted as previously described (8). Equal amounts of protein (10 μ g) were loaded and separated on 11% SDS-PAGE gels, followed by transfer to polyvinylidene fluoride membranes. Following 30 min incubation with blocking buffer (ZLI 9027; ZSGB-BIO; OriGene Technologies, Inc., Beijing, China), composed of 5% bovine serum albumin in TBS with 0.05% Tween-20, membranes were probed with antibodies against NLRP3, APN, AdipoR1, AMPK α , pAMPK α , TNF α and GAPDH at 4°C overnight. Immune complexes were visualized following incubation with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies for 1 h at room temperature. Immunoreactive bands were detected using enhanced chemiluminescence (Amersham; GE Healthcare, Chicago, IL, USA). Signals were quantified using the Tanon-4500 gel imaging system with GIS ID analysis software v4.1.5 (Tanon Science and Technology Co., Ltd., Shanghai, China).

APN, IL-1 β and TNF α levels in cell supernatants. Growth-arrested PTECs (5×10^5) were incubated with 100 μ g/ml SUA for 48 h as previously described (8), with or without pretreatment with AdipoR1-shRNA lentivirus for 24 h or 10 mM compound C for 90 min. Cell supernatants were collected. APN levels were measured at 450 nm with a high sensitivity (<60 pg/ml) ELISA kit (cat. no. EK0595; Boster Biological Technology, Pleasanton, CA, USA). The standard provided with the kit is a 30 kDa APN protein, representing a full-length APN. IL-1 β and TNF α were quantified using commercial ELISA kits at 450 nm. The sensitivity of the TNF α assay was 0.13 pg/ml and its intra- and interassay coefficients of variation were 8.5 and 9.8%, respectively. The sensitivity and intra- and interassay coefficients of variation for the IL-1 β assay were 0.05 pg/ml, 6.7 and 8.1%, respectively.

Statistical analysis. Statistical analyses were performed using SPSS 21.0 software (IBM Corp., Armonk, NY, USA). The results are expressed as the mean \pm standard errors of the mean. Comparisons between two groups were analyzed by a two-tailed Student's t-test. Comparisons among three or more groups were analyzed by one-way analysis of variance followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

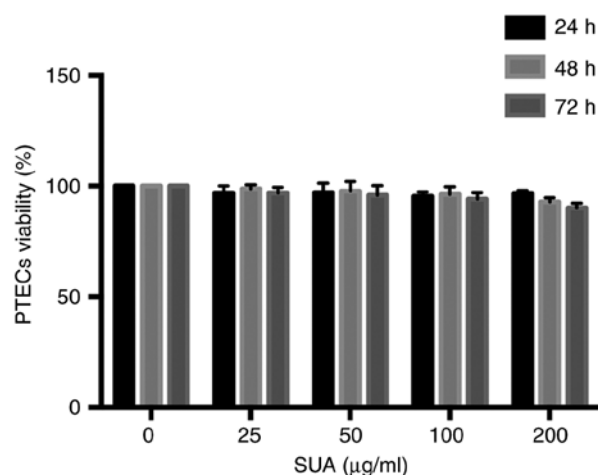


Figure 1. Viability of PTECs exposed to SUA. PTECs were treated with SUA at 0, 25, 50, 100 and 200 μ g/ml for 24, 48 and 72 h (n=3). Cell viability was assessed using MTT assays and results are presented as the mean \pm standard error the mean of independent experiments vs. untreated cells defined as 100%. PTEC, proximal renal tubular epithelial cell; SUA, soluble uric acid.

Results

Effect of SUA on cultured human PTEC viability. To determine whether SUA impaired cell viability, increasing doses of SUA (25–200 μ g/ml) were added to PTECs for varying time periods (24–72 h). Cell viability was assessed using an MTT assay and expressed as the percentage change in the absorbance relative to untreated PTECs. Fig. 1 revealed that SUA administration did not significantly affect cell viability at any of the indicated time points ($P > 0.05$). A dose of 100 μ g/ml SUA, corresponding to a common level of UA in hyperuricemia in humans, was employed in the following experiment (33).

Inflammatory responses are elevated in SUA-treated PTECs. The impact of SUA on inflammatory responses was evaluated in cultured PTECs. PTECs were incubated in the presence of absence of 100 μ g/ml SUA for 48 h. SUA treatment significantly enhanced the protein synthesis of NLRP3 and TNF α in PTECs compared with the untreated control ($P < 0.001$ and $P < 0.05$, respectively; Fig. 2A–C). SUA further significantly promoted the release of IL-1 β ($P < 0.01$; Fig. 2D) and TNF α from PTECs into the serum compared with the untreated control ($P < 0.001$; Fig. 2E).

APN expression is increased in SUA-treated PTECs. Effects of SUA on the expression of APN in cultured PTECs were established. PTECs were incubated in the presence or absence of 100 μ g/ml SUA for indicated time periods. SUA treatment significantly increased APN mRNA expression following a 4-h incubation and protein expression following a 48-h incubation ($P < 0.05$; Fig. 3A and B).

APN release was increased in SUA-treated PTECs compared with the untreated control (0.23 ± 0.09 vs. 0.07 ± 0.03 μ g/ml; $P = 0.16$; Fig. 3C). To analyze whether APN treatment attenuates SUA-induced inflammation responses in PTECs, cells were cultured in the presence or absence of 2.5 μ g/ml gAPN for 6 h prior to incubation with 100 μ g/ml SUA for 48 h. Exogenous gAPN inhibited the SUA-induced

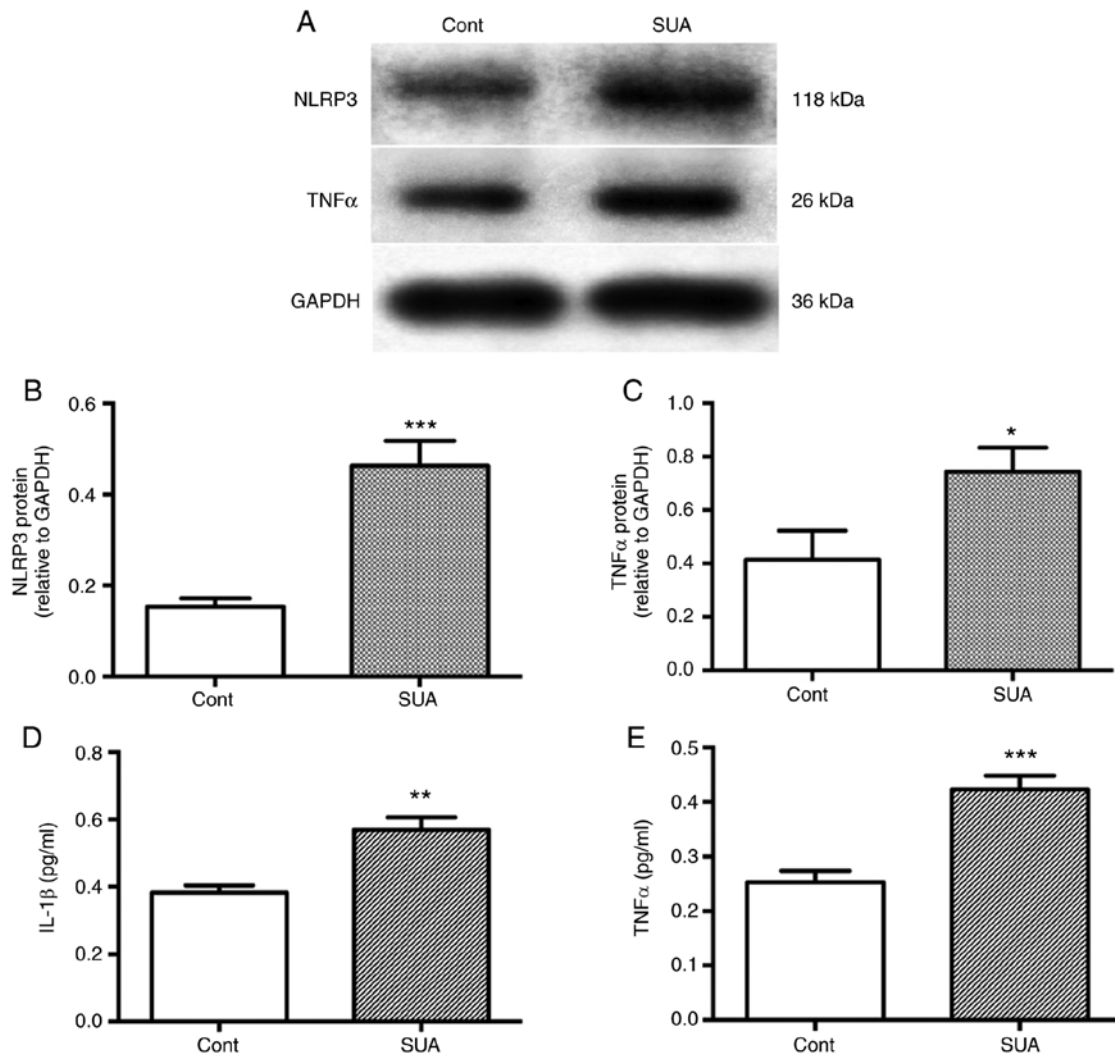


Figure 2. SUA provokes an inflammatory reaction in PTECs. PTECs were incubated in the presence and absence of SUA (100 μ g/ml) for 48 h (n=3). (A) Western blot images and quantified protein expression of (B) NLRP3 and (C) TNF α . Levels of (D) IL-1 β and (E) TNF α in cell supernatants measured using ELISA. Data are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. Cont. PTEC, proximal renal tubular epithelial cell; SUA, soluble uric acid; NLRP3, NACHT, leucine rich repeat and pyrin domain-containing protein 3; TNF, tumor necrosis factor; IL, interleukin; Cont, untreated cells.

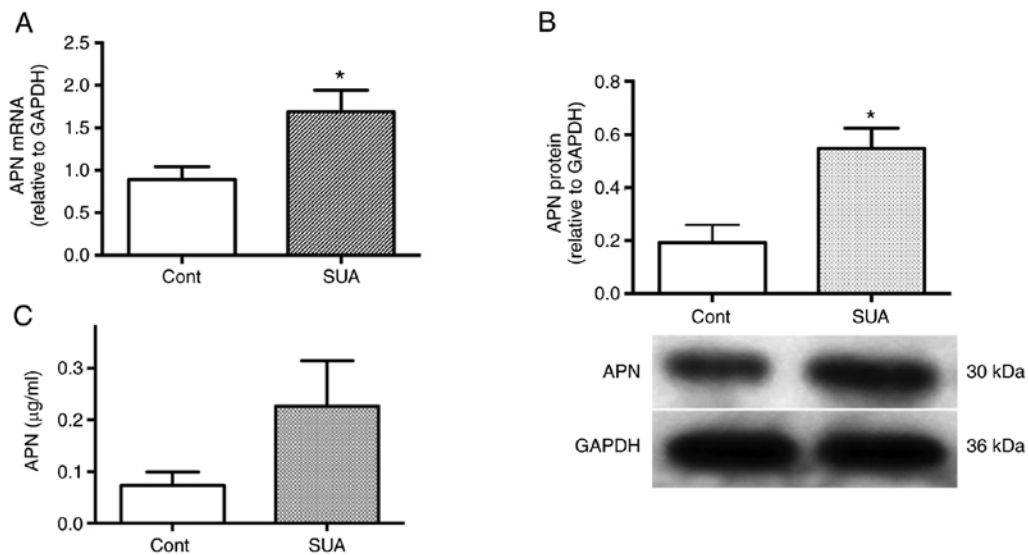


Figure 3. SUA stimulates APN expression in PTECs. PTECs were incubated in the presence and absence of SUA (100 μ g/ml; n=3). (A) mRNA and (B) protein expression following 4 and 48 h incubation, respectively. (C) Secreted APN levels were determined using ELISA following a 48 h incubation. Data are representative of three independent experiments. *P<0.05 vs. Cont. PTEC, proximal renal tubular epithelial cell; SUA, soluble uric acid; APN, adiponectin; Cont, untreated cells.

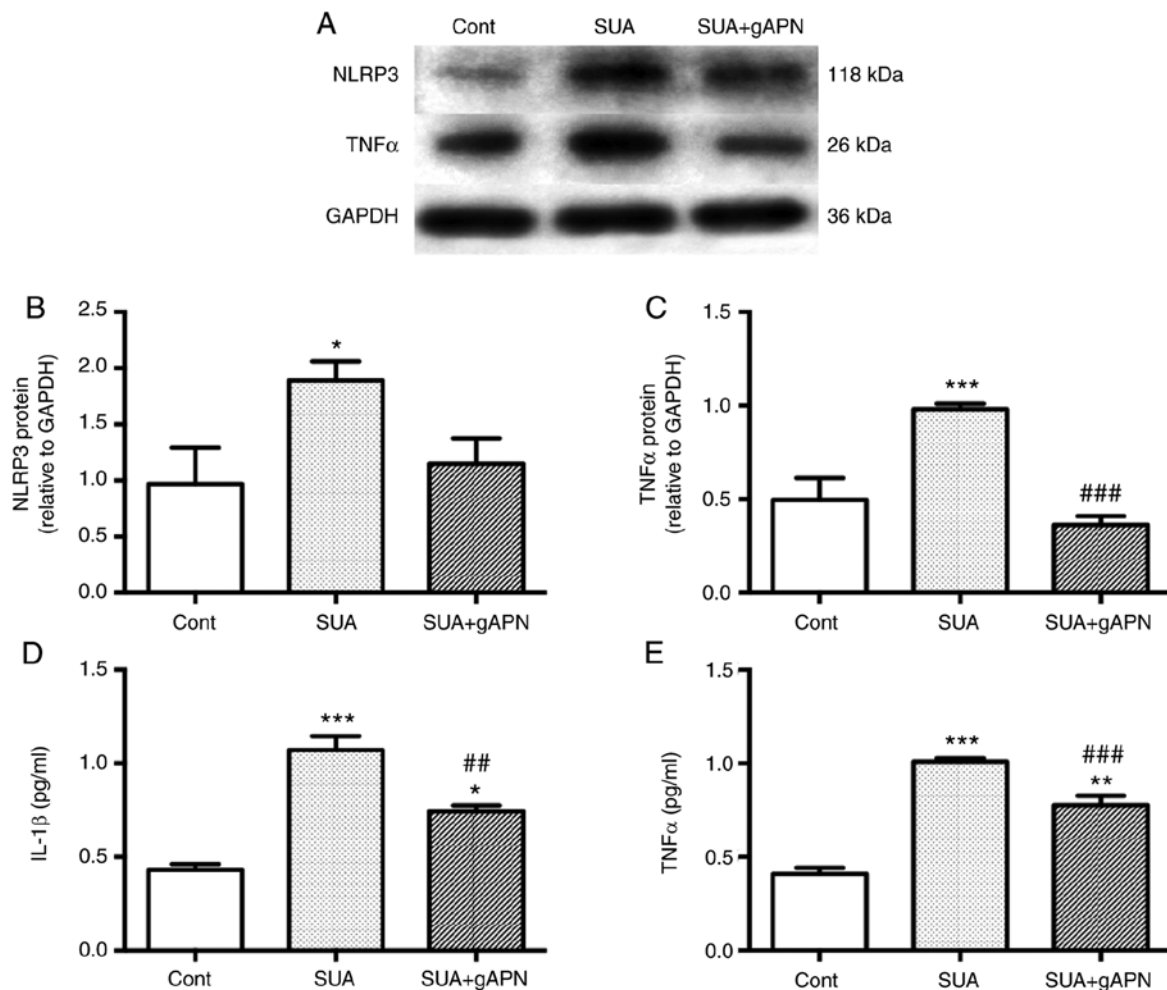


Figure 4. APN supplement alleviates SUA-triggered inflammatory responses in PTECs. PTECs were pretreated gAPN (0 or 2.5 $\mu\text{g/ml}$) for 6 h prior to incubation with SUA (100 $\mu\text{g/ml}$) for 48 h (n=3). (A) Western blot images and quantified protein expression of (B) NLRP3 and (C) TNF α . Levels of (D) IL-1 β and (E) TNF α in cell supernatants measured using ELISA. Data are representative of three independent experiments. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs. Cont; ## $P<0.01$ and ### $P<0.001$ vs. SUA. APN, adiponectin; g, globular; PTEC, proximal renal tubular epithelial cell; SUA, soluble uric acid; NLRP3, NACHT, leucine rich repeat and pyrin domain-containing protein 3; TNF, tumor necrosis factor; IL, interleukin; Cont, untreated cells.

elevation of cellular and secreted protein levels (Fig. 4). TNF α levels were significantly decreased in gAPN-pretreated cells compared with the SUA-treated cells as determined by western blotting and ELISA analysis ($P<0.001$; Fig. 4A, C and E). gAPN administration further significantly decreased IL-1 β levels in the supernatant of PTECs incubated with SUA ($P<0.01$; Fig. 4D). No significant changes in NLRP3 protein expression were observed in SUA-treated cells pretreated with APN ($P>0.05$; Fig. 4A and B). The data indicated that APN may inhibit SUA-induced inflammation *in vitro* and may aid in restoring the balance between anti- and proinflammatory environments.

APN activates the AdipoR1/AMPK signaling pathway in PTECs. To investigate whether the AdipoR1/AMPK signaling pathway is involved in the anti-inflammatory mechanism of gAPN, PTECs were treated with 2.5 $\mu\text{g/ml}$ gAPN for 6 h followed by incubation with 100 $\mu\text{g/ml}$ SUA for 48 h. AdipoR1 protein expression and AMPK phosphorylation at threonine-172 were assessed. The results suggested that SUA significantly increased AdipoR1 protein expression and AMPK phosphorylation in PTECs compared with the

untreated control ($P<0.05$ and $P<0.01$, respectively; Fig. 5). gAPN treatment further significantly increased AdipoR1 protein and AMPK phosphorylation levels compared with the SUA-treated cells ($P<0.01$ and $P<0.05$, respectively).

AdipoR1 knockdown amplifies SUA-induced inflammatory responses in PTECs. To clarify whether AdipoR1 is involved in the anti-inflammatory effects of APN, PTECs were subjected to 24 h transfection with AdipoR1-shRNA or scramble-shRNA prior to SUA treatment (100 $\mu\text{g/ml}$) for 48 h. AdipoR1-shRNA transfection significantly decreased AdipoR1 mRNA and protein levels compared with the untreated control by 32.33 and 61.45% ($P<0.05$ and $P<0.001$, respectively; Fig. 6A and B). No significant difference between the scramble-shRNA and the AdipoR1-shRNA was detected at mRNA level ($P>0.05$); however, protein levels were significantly different ($P<0.001$). AdipoR1-shRNA transfection markedly increased NLRP3 protein levels compared with the SUA-treated cells ($P>0.05$; Fig. 6C and D). AdipoR1 knockdown significantly increased synthesis and secretion of TNF α ($P<0.05$ and $P<0.001$, respectively; Fig. 6C, E and G) and increased IL-1 β secretion from cultured PTECs

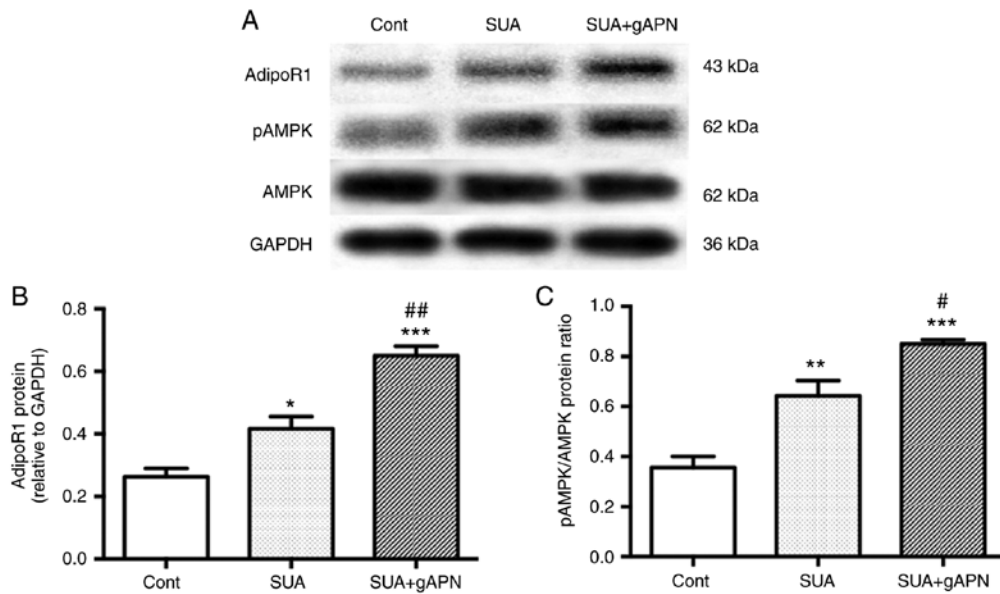


Figure 5. APN supplement activates the AdipoR1/AMPK signaling pathway in PTECs incubated with SUA. PTECs were pretreated gAPN (0 or 2.5 μ g/ml) for 6 h prior to incubation with SUA (100 μ g/ml) for 48 h (n=3). (A) Western blot images and quantified levels of (B) AdipoR1 and (C) AMPK phosphorylation. Data are representative of three independent experiments. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs. Cont; # $P<0.05$ and ## $P<0.01$ vs. SUA. APN, adiponectin; g, globular; PTEC, proximal renal tubular epithelial cell; SUA, soluble uric acid; AdipoR1, APN receptor 1; AMPK, adenosine monophosphate-activated protein kinases; p, phosphorylated; Cont, untreated cells.

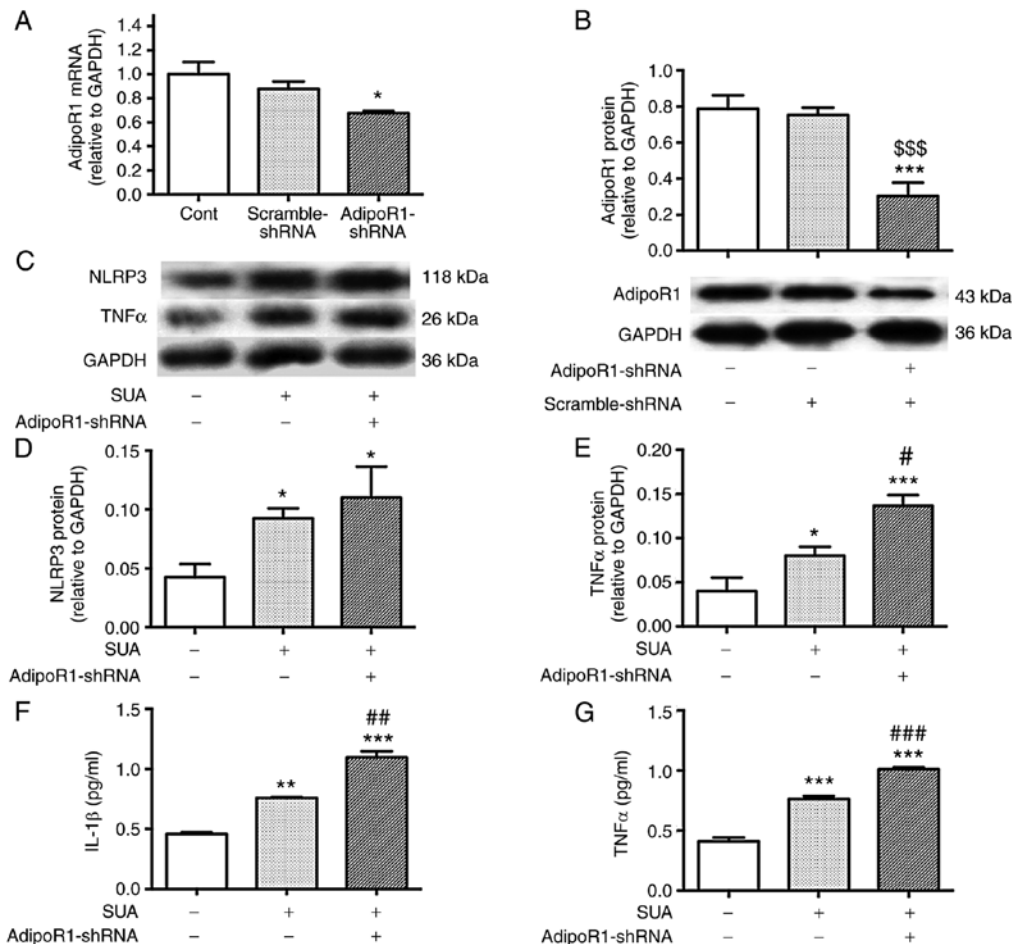


Figure 6. AdipoR1 knockdown promotes inflammation in PTECs induced by SUA. PTECs were transfected with AdipoR1-shRNA or scramble-shRNA prior to treatment with SUA (100 μ g/ml) for 48 h (n=3). AdipoR1 (A) mRNA and (B) protein expression in shRNA transfected cells. (C) Western blot images and quantified protein expression of (D) NLRP3 and (E) TNF α . Levels of (F) IL-1 β and (G) TNF α in cell supernatants measured using ELISA. Data are representative of three independent experiments. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs. Cont; # $P<0.05$, ## $P<0.01$ and ### $P<0.001$ vs. SUA; \$\$\$ $P<0.001$ vs. scramble-shRNA. APN, adiponectin; PTEC, proximal renal tubular epithelial cell; SUA, soluble uric acid; NLRP3, NACHT, leucine rich repeat and pyrin domain-containing protein 3; TNF, tumor necrosis factor; IL, interleukin; Cont, untreated cells; sh, short hairpin; AdipoR1, APN receptor 1.

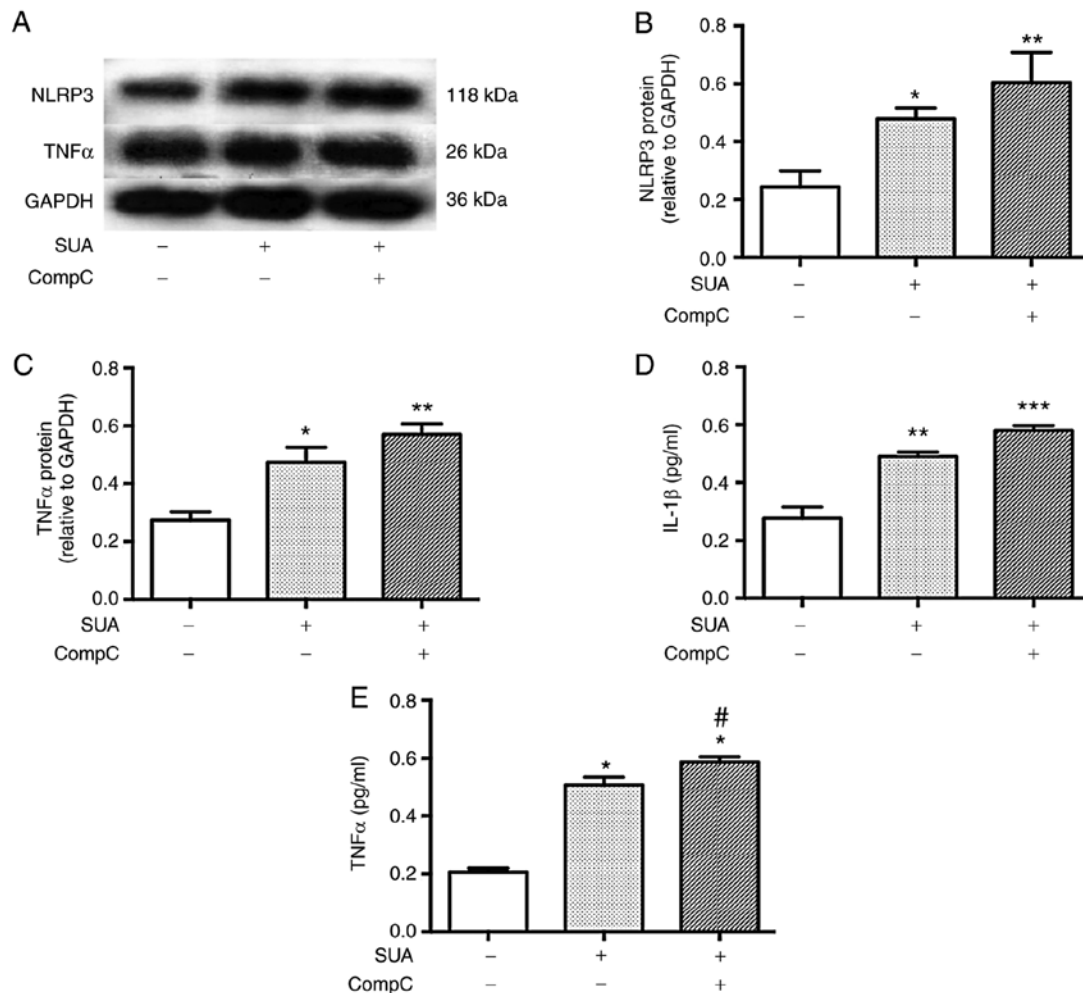


Figure 7. AMPK inhibitor aggravates SUA-induced inflammation in PTECs. PTECs were preincubated with compound C (10 μ M) for 90 min prior to stimulation with SUA (100 μ g/ml) for 48 h (n=3). (A) Western blot images and quantified protein expression of (B) NLRP3 and (C) TNF α . Levels of (D) IL-1 β and (E) TNF α in cell supernatants measured using ELISA. Data are representative of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. Cont; and #P<0.05 vs. SUA. APN, adiponectin; PTEC, proximal renal tubular epithelial cell; SUA, soluble uric acid; NLRP3, NACHT, leucine rich repeat and pyrin domain-containing protein 3; TNF, tumor necrosis factor; IL, interleukin; Cont, untreated cells; sh, short hairpin; AdipoR1, APN receptor 1; CompC, compound C; AMPK, adenosine monophosphate-activated protein kinases.

compared with the SUA-treated cells (P<0.01; Fig. 6F). The data indicated that AdipoR1 knockdown further promoted inflammatory responses induced by SUA in PTECs.

AMPK inhibitor promotes SUA-induced TNF α secretion from PTECs. To test whether AMPK signaling was associated with APN-induced resistance to SUA-induced inflammation, PTECs were subjected to incubation with compound C (10 mM), a specific AMPK inhibitor, for 90 min followed by exposure to SUA (100 μ g/ml) for 48 h. Compound C administration further promoted the SUA-induced inflammatory responses (Fig. 7). Protein expression of NLRP3 and TNF α was slightly increased by the inhibitor treatment compared with the SUA-treated cells (P>0.05; Fig. 7A-C). Secretion of IL-1 β was further markedly increased by the inhibitor treatment (P>0.05; Fig. 7D), while TNF α secretion exhibited a significant increase in the compound C treated cells compared with the SUA-treated cells (P<0.05; Fig. 7E). The data indicated that an AMPK inhibitor partially increased the proinflammatory reaction induced by SUA in cultured PTECs.

Discussion

The present study demonstrated that adiponectin (APN) induced a resistance to SUA-triggered inflammation in PTECs. APN supplementation suppressed SUA-induced inflammatory mediators partially via the AdipoR1/AMPK signaling pathway. Both AdipoR1 knockdown and AMPK inhibition intensified the SUA-induced release of proinflammatory cytokines. To the best of our knowledge, this report is the first report describing regulatory function of APN in renal tubular injury in the context of SUA stimulation. The findings of the present study may assist in clarifying the pathogenesis of SUA-associated renal disease.

Previous studies have revealed that SUA triggers renal inflammation (8,34) by activating the NLRP3/IL-1 β signaling pathway (8) and amplifying TNF α mRNA expression (7) as a result of nuclear factor (NF)- κ B activation (35,36). However, the mechanism by which tubular inflammation may be inhibited is poorly understood. In the present study, it was observed that APN supplementation alleviated the production and release of TNF α in SUA-treated PTECs, similar to the findings described

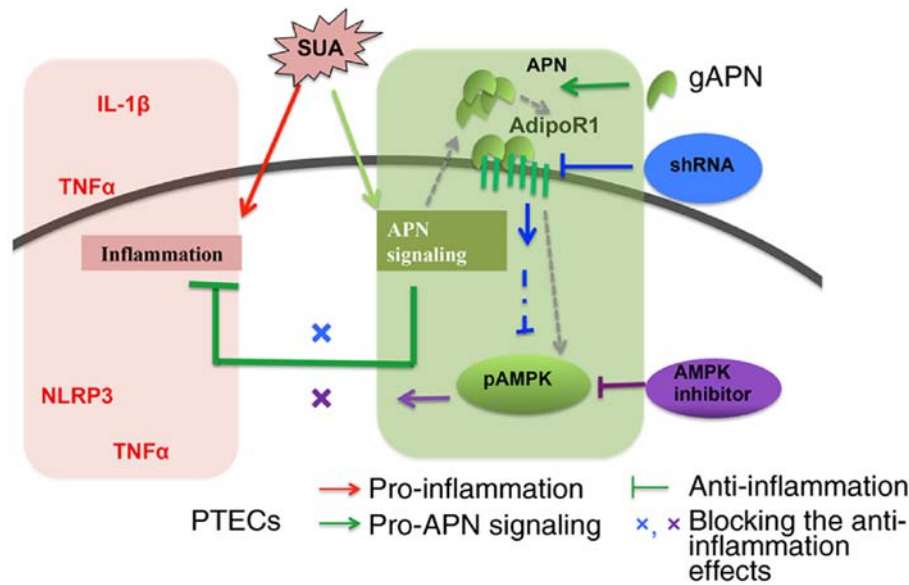


Figure 8. Proposed model for APN inhibition of inflammatory responses in PTECs induced by SUA. SUA evokes inflammatory responses and induces protein expression of NLRP3 and TNF α , and the release of TNF α and IL-1 β . APN, upregulated by SUA, inhibits expression of inflammatory factors and promotes AdipoR1 synthesis and AMPK activation. AdipoR1 knockdown and AMPK inhibition increase proinflammatory mediators. APN, adiponectin; g, globular; AdipoR1, ANP receptor 1; PTEC, proximal renal tubular epithelial cell; SUA, soluble uric acid; NLRP3, NACHT, leucine rich repeat and pyrin domain-containing protein 3; TNF, tumor necrosis factor; IL, interleukin; g, globular; AMPK, adenosine monophosphate-activated protein kinases; p, phosphorylated; sh, short hairpin.

for cardiomyocytes, adipocytes (37) and macrophages (38,39). APN was reported to decrease TNF α production in myocytes under ischemia-reperfusion or LPS exposure (36,37,39) and APN deficiency increases TNF α levels in ischemic-reperfused heart tissue (37). Accumulating evidence has demonstrated that APN exerts renoprotective effects through inhibiting NF- κ B activity in high glucose- or TNF α -treated renal mesangial cells (28) and alleviating NF- κ B-associated inflammatory responses induced by angiotensin II (27). In contrast, APN depletion exacerbates inflammatory damage and upregulates the intrarenal levels of proinflammatory factors in diabetic (28) and subtotal nephrectomized mice (25). TNF α stimulates APN expression, which in turn increases IL-8 release via AdipoR1, in lung epithelial cells (40). These findings indicate that TNF α induces an APN-AdipoR1-dependent proinflammatory effect in lungs (40). Conversely, numerous studies have indicated that APN exerts anti-inflammatory effects via suppression of TNF α synthesis and induction of anti-inflammatory cytokines (41-43). These observations are consistent with the findings of the present study suggesting that gAPN suppressed the release of TNF α and IL-1 β from SUA-treated PTECs. Unlike results presented by Miller *et al* (40), TNF α and APN levels were simultaneously increased in SUA-treated PTECs compared with the untreated control. The regulatory effect of APN on inflammatory factors may depend on the targeted cell type, dose and the APN stimulator. It is hypothesized that SUA-stimulated TNF α amplifies APN expression via a positive feedback loop and overexpressed APN reacts to alleviate inflammation. Direct evidence of TNF α -induced APN expression in SUA-treated renal cells requires further elucidation.

IL-1 β induces proinflammatory activity (44). Maturation of IL-1 β is controlled by the NF- κ B-dependent production and the NLRP3 inflammasome-dependent proteolytic processing of pro-IL-1 β (45). Notably, it was observed

that gAPN administration inhibited IL-1 β release without significantly influencing NLRP3 production. IL-1 β may be an adaptor molecule of the NLRP3 inflammasome complex, similar to apoptosis-associated speck-like protein containing a caspase recruitment domain or caspase-1, instead of an NLRP3-controlled maturation of IL-1 β in SUA-exposed PTECs. Current findings indicated a potent anti-inflammatory function of APN in SUA-exposed PTECs.

Cellular APN secreted from HK-2 cells (<2 ng/ml) (19) and PTECs (70 ng/ml; present study) was decreased compared with plasma APN from healthy volunteers (1.9 to 17.0 mg/ml) (46). Findings of the present study demonstrated that SUA increased inflammatory cytokine expression in the presence of endogenous APN in PTECs, similar to the lipopolysaccharide-induced NF- κ B upregulation in HK-2 cells observed previously (29). Anti-inflammatory effects of APN were determined in the present study for gAPN supplementation at a dose of 2.5 μ g/ml; this dose is increased compared with the endogenous APN concentration determined previously (<2 ng/ml) (19). Findings suggested a dose-associated biological effect of APN and clarify why a moderate amount of endogenous APN, 70 ng/ml in the present study, did not inhibit SUA-induced inflammation. These comparisons may be regarded with caution due to the data originating from various cell systems with dissimilar APN doses and treatment durations. Future experiments may investigate whether PTECs produce elastases involved in cleaving APN and generating its globular domain at the amino-terminal collagenous domain as suggested previously (47). Further research is required to analyze whether UA further affects these processes.

AdipoR1 and AdipoR2 are well-known APN receptors, which have been detected in renal tubular cells (19,48). AdipoR1 levels are increased compared with AdipoR2 (48), suggesting a superior function association with APN. A previous study

on 3T3-L1 preadipocytes demonstrated distinctly increased mRNA levels of AdipoR1 compared with AdipoR2 (49). AdipoR1 exhibits a high affinity for gAPN (23) and contributes to the phosphorylation and activation of AMPK (23,50,51). In the present study effects of gAPN treatment on AdipoR1 expression were evaluated. gAPN upregulated AdipoR1 protein expression while reducing synthesis and secretion of inflammatory mediators in SUA-treated PTECs. These data indicated that AdipoR1 may be involved in the resistance conferred by APN to SUA-induced renal tubular inflammation and may advice the potential use of AdipoR1 in urate nephropathy.

AMPK, a sensitive energy sensor, participates in various pathophysiological processes and mediates beneficial actions of APN (18,20,22,37). It was demonstrated that AMPK activity is decreased in the hearts of APN-KO mice (37). AMPK deficiency further abrogated antiapoptotic activities of APN in cardiomyocytes following hypoxia-reoxygenation (37). The current research demonstrated that gAPN administration limited inflammatory responses and enhanced AMPK phosphorylation in SUA-treated PTECs, suggesting a positive involvement of AMPK in APN-mediated anti-inflammatory processes. Taken together, these findings imply that AMPK may mediate renoprotective effects of APN (52-54). Future studies are required to investigate the effect of gAPN-AMPK inhibitor co-treatment on PTECs to determine whether treatment with an AMPK-inhibitor abolishes APN-induced inhibition of inflammation following SUA treatment. A gene KO procedure may be performed to support conclusions drawn from reports of AMPK-independent effects of compound C in various cells (55,56). The low efficiency of shRNA transfections in the present study may be the reason for the inability of the AdipoR1-shRNA to fully suppress AMPK activity. The presence of AdipoR2 (19,48) may provide an additional explanation for minor changes in AMPK phosphorylation.

IR is essential in hyperuricemia-induced renal tubular nephrolithiasis (11,12). Furthermore, abnormalities in APN signaling and IR affect each other. Hypoadiponectinemia contributes to diabetes and metabolic syndrome (16), which are characterized by IR. Conversely, IR impairs AdipoR1 transcription (17) and AMPK activation, decreasing the response sensitivity of APN (16). APN/AdipoR1/AMPK signaling pathway disruptions may partially describe physiological conditions associated with IR. Therefore, the present study mimicked IR by treatment with AdipoR1-shRNA and AMPK inhibitor, which augmented the release of inflammatory cytokines from SUA-treated PTECs. The results suggested that abnormal APN signaling and IR may increase the vulnerability of PTECs to SUA-induced inflammation. However, the present study was a preliminary *in vitro* study and did not provide direct data on IR. Future investigations using a hyperuricemic model may evaluate the extent of IR and potential AMPK targets in AdipoR1 and AMPK KO cells. Additionally, interventions in a hyperuricemic animal model with and without IR may be performed to elucidate whether alleviating IR ameliorates UA-induced renal tubular inflammatory injury.

Several limitations still exist in the present study. AdipoR1 signaling differentially modulated protein expression of TNF α , IL-1 β and NLRP3. This raises the question whether

other signaling pathways are involved in the regulation of TNF α and whether alternative receptors for APN, including AdipoR2 or T-cadherin (57), are involved in the regulation of NLRP3 expression. There was a non-significant difference at AdipoR1 mRNA levels between the scramble- and the AdipoR1-shRNA. This may be partially associated with off-target effects. Further study will focus on modifying the shRNA design to improve the knockdown efficiency. Furthermore, the present study did not explore if alternative components of the mature NLRP3 inflammasome were involved in anti-inflammatory effects exerted by APN. Further study will address this question using RT-qPCR and western blot analysis. Additionally, control experiments studying PTECs pretreated with APN in the absence of SUA were not performed and therefore, direct effects of APN on AMPK or inflammatory factors, as previously described, were not evaluated here (27,39). The underlying mechanism by which APN is upregulated in SUA-treated PTECs remains to be elucidated.

In summary, the present study indicated that APN exerted protective effects against SUA-induced inflammation in PTECs at least partially via the AdipoR1/AMPK signaling pathway (Fig. 8).

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZY and QY conceived and designed the experiments. QY and JX performed the experiments. CF and XZ analyzed the data. QY, JZ and ZZ interpreted data and prepared the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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