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The Rho kinase signaling pathway participates in tubular mitochondrial oxidative injury and apoptosis in uric acid nephropathy

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Abstract

Introduction: Oxidative stress is a pathologic feature of hyperuricemia that is highly prevalent and that contributes to kidney tubular interstitial fibrosis. Rho-kinase is closely related to mitochondrial-induced oxidative stress. Herein, we designed a study to explore the expression and role of Rho-kinase in hyperuricemia nephropathy. The secondary objective was to investigate whether the Rho-kinase signaling pathway regulates hyperuricemic tubular oxidative injury and apoptosis via the mitochondrial pathway in addition to the mechanisms that are involved.

Materials and methods: HK-2 cells were divided into the following five groups: normal; uric acid (UA); UA+Fasudil; UA+ROCK1 si-RNA; and UA+sc-siRNA. Rho-kinase activity, mitochondrial oxidative injury, and apoptosis-related protein levels were measured in each group. A *t*-test was used to analyze the difference between groups.

Results: Myosin phosphatase target subunit 1 (MYPT1) overexpression was shown in HK-2 cells, which was caused by UA. High concentrations of UA also up-regulated Rho-kinase expression and mitochondrial and apoptosis-related protein expression, while treatment with fasudil and ROCK1 si-RNA significantly attenuated these responses.

Conclusion: The Rho-kinase signaling pathway participates in tubular mitochondrial oxidative injury and apoptosis via regulating mitochondrial dyneins/biogenic genes in UA nephropathy,

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which suggests that the mitochondrial pathway might be a potential therapeutic target for hyperuricemia nephropathy.

Keywords
Hyperuricemia, Rho kinase, signaling pathway, tubular mitochondrial oxidative injury, uric acid nephropathy, dynein, gene regulation

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Introduction

Increased uric acid is an important clinical feature for many diseases. Hyperuricemia occurs because of increased serum uric acid production or under-excretion of uric acid in the urine.[1] Renal tubular epithelial cells can reabsorb water, amino acids, glucose, electrolytes, and urea.[2] When renal tubular epithelial cells are impaired, the effective reabsorption function cannot occur, leading to abnormal physiological activity and to chronic kidney disease (CKD). CKD is defined as the presence of renal damage or renal dysfunction for more than 3 months,[3] and evidence showed that hyperuricemia is associated with renal tubular epithelial cell injury, which increases the risk of CKD.[4–6] Studies showed that hyperuricemia impaired renal function occurs via obstruction of renal tubules by urate crystal deposition, which results in renal tubular cell injury and subsequent renal interstitial fibrosis.[7,8] Therefore, early stage intervention measures for hyperuricemia nephropathy (HN) are of great importance to reduce the socioeconomic burden of CKD.

Various studies have shown that the development and progression of HN can occur through multiple mechanisms that are independent of acid crystal formation, such as promoting autophagy and NLRP3-mediated inflammation,[9,10] inhibiting transforming growth factor (TGF)-β, extracellular signal-regulated kinase 1/2 (ERK1/2), and nuclear factor (NF)-κB signaling pathways.[11] Hyperuricemia can cause oxidative stress that induces the overproduction of mitochondrial reactive oxygen species (ROS) in renal tubular epithelial cells, which play an important role in the pathogenesis of HN tubulointerstitial fibrosis.[12] Oxidative stress that is generated by monosodium urate (MSU) crystals promotes renal cell apoptosis, which is induced through the mitochondrial caspase-dependent apoptosis pathway.[13]

As an effector of small G protein Rho, Rho kinase has many important physiological and pathological functions, including cytoskeletal reorganization, cell migration, apoptosis, and gene expression.[14] An increasing amount of evidence indicates that excessive activation of the Rho kinase signaling pathway may be involved in chronic fibrosis of the myocardium, lung, and kidney. Recently, we showed that high glucose exposure induced activation of renal interstitial fibroblasts through the Rho kinase signal pathway, while the damage caused by mitochondrial oxidative and renal tubular epithelial cell apoptosis was blocked by treatment with fasudil, which targets the ATP-dependent kinase domain and inhibits ROCK.[15] In addition, as an inhibitor of Rho kinase, fasudil attenuates both cyclosporine-induced kidney interstitial fibrosis and the parathormone-induced renal proximal tubular cell epithelial-to-mesenchymal transition (EMT).[16,17] These studies indicate that Rho kinase is coupled to the onset and progression of tubulointerstitial fibrosis,

which are associated with mitochondrial-induced oxidative stress. However, it remains unclear whether Rho kinase is involved in the progression of HN through the mitochondrial pathway. Myosin phosphatase target subunit 1 (MYPT1) is phosphorylated at multiple sites by several kinases, and therefore, the level of phosphorylated MYPT1 can be an indicator of ROCK activation. Dynamin-related protein 1 (Drp1) is essential for segregation of damaged mitochondria for degradation, and mitofusin 1 (Mfn1) is required for mitochondrial fusion and for mitochondria–endoplasmic reticulum interaction. Peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1α) and nuclear respiratory factor-1 (NRF-1) are involved in mitochondrial biogenesis. When the mitochondria are exposed to ROS, caspase-9 is activated, and therefore, caspase-3 is activated, which further induces apoptosis.

The purpose of this investigation was to determine the effect of Rho kinase on HN. We also investigated whether the Rho kinase signaling pathway regulates high uric acid-induced tubular oxidative injury and apoptosis though the mitochondrial pathway. This study suggests a safer and more effective therapeutic target for HN.

Materials and methods

Cell culture

Renal tubular epithelial cells (HK-2 cells) were obtained from the cell bank at the Central Experiment Laboratory (Xiangya, China), and the cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and in DMEM/F12 medium supplemented with 10% FBS and 1% P/S. At 50% to 60% confluence, the growth medium was replaced with serum-free medium for 24 hours to arrest growth. Thereafter, the cells were treated with the different protocols, as follows: (1) normal control group; (2) HK-2 cells were treated with 5 ng/mL TGF-β1; (3) HK-2 cells were treated with 400 μM uric acid for 48 hours; or (4) HK-2 cells were pre-treated with 10 μmol/L fasudil for 30 minutes, then cultured with 400 μM uric acid for 48 hours; and (5) HK-2 cells were transfected with ROCK1 small interfering (si)RNA or scrambled (sc)-siRNA before treatment with 400 μM uric acid. The experimental method using siRNA or sc-siRNA was described by Du et al.[18] Cells were harvested and used for immunoblotting and reverse transcription polymerase chain reaction (RT-PCR).

Reverse transcription polymerase chain reaction

Total RNA was extracted from HK-2 cell lysates using Trizol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. Reverse transcription was conducted using the Superscript III RT kit (Invitrogen), and the protocols were conducted in accordance with the manufacturer's instructions. Primers were synthesized by Invitrogen (Table 1). Reaction cycling conditions were as follows: 35 cycles of 94°C for 5 minutes, 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s, followed by 72°C for 5 minutes.

Western blot analysis

Western blotting was conducted on protein extractions that were obtained from the cell lysates. The protein concentrations were measured using a bicinchoninic acid (BCA) kit. Lysate proteins were separated using 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred electrophoretically to a nitrocellulose membrane. The membranes were blocked for 1 hour at room temperature using 5% nonfat milk in tris-buffered saline (TBS), then incubated at 4°C overnight with the following primary antibodies: anti-MYPT1 (1:1000; this and all

Table 1. Primers used in this study

Primers		Sequence
Drp1 (198 bp)	F	5′-GGGGTTGGAGATGGTG-3′
	R	5′-CTGTTCCCGAGCAGATAG-3′
PGC-1α (173 bp)	F	5′-GACGACGAAGCAGACAAG-3′
	R	5′-GAATAGGTTGCGTGCC-3′
MnSOD2 (197 bp)	F	5′-GGGTTGGCTTGGTTC-3′
	R	5′-CATTCTCCCAGTTGATTACA-3′
β-actin (231 bp)	F	5′-ACTCTTCCAGCCTTCCTTCC-3′
	R	5′-GAGGAGCAATGATCTTGATCTTC-3′

DRP1, dynamin-related protein 1; PGC-1α, peroxisome proliferator activated receptor gamma coactivator 1 alpha; MnSOD2, manganese superoxide dismutase; F, forward; R, reverse.

subsequent antibodies were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-Drp1 (1:1000), anti-Mfn1 (1:1000), anti-PGC-1α (1:1000), anti-NRF-1 (1:1000), anti-pro-caspase 3 (1:1000), anti-pro-caspase 9 (1:1000), and beta-actin (1:1000). After washing in TBS-Tween20, the membranes were incubated with secondary antibodies for 2 hours at room temperature. After washing, the membranes were incubated with enhanced chemiluminescence (ECL) reagents and scanned using a Bio-Rad Electrophoresis Image Analyzer (Bio-Rad, Hemel Hampstead, UK).[19,20]

Statistical analysis

All results are expressed as the mean ± standard deviation (SD) and analyzed using SPSS 20.0 (IBM Corp., Armonk, NY, USA). Data were calculated for at least three independent experiments. An unpaired Student's *t*-test was used to detect the differences between two groups. $P<0.05$ was defined as a statistically significant difference.

Results

Rho kinase activity in high uric acid-induced HK-2 cells

To confirm the effect of uric acid on Rho kinase activity, we performed an *in vitro* study in HK-2 cells. Exposure of HK-2 cells to 400 μM uric acid resulted in an upregulation of MYPT1, which is a measure of Rho kinase activity. This was similar to the effect of HK-2 cells that were incubated with 5 ng/mL TGF-β1 (Figure 1). Thus, we suggest that the Rho kinase signaling pathway may contribute to the high uric acid level that induced Rho kinase activation in HK-2 cells.

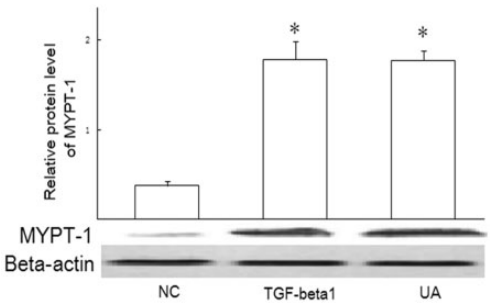

Figure 1. Upregulation of MYPT1 protein in HK-2 cells induced by high uric acid, which indicates that the Rho kinase signal pathway was involved in high uric acid-induced Rho-kinase activation in HK-2 cells. HK-2 cells were incubated with 5 ng/mL of TGF-β1 or 400 μM uric acid (n=3). Western blots showed that treatment with 400 μM uric acid significantly increased the MYPT1 protein expression. *, $P<0.01$ vs. normal control group; #, $P=0.01$ vs. 400 μM uric acid group.
MYPT1, myosin phosphatase target subunit 1; TGF, transforming growth factor.

Effects of the Rho kinase pathway on mitochondria-induced oxidative damage induced by high uric acid in HK-2 cells

Uric acid can trigger oxidative stress, but it is unclear whether the Rho kinase pathway mediates uric acid-induced oxidative damage to the mitochondria. As shown in Figure 2, uric acid markedly up-regulated the transcription of manganese superoxide dismutase (MnSOD2) by RT-PCR ($P<0.01$ vs. NC group). Pre-treatment with fasudil (10 μmol/L) significantly blocked uric acid-induced changes in MnSOD2 transcription in HK-2 cells ($P<0.05$ vs. uric acid-treated cells). Additionally, a significant decrease in MnSOD2 mRNA expression was observed in HK-2 cells that were transfected with ROCK1 si-RNA, while this effect did not occur in the sc-siRNA group. These findings suggested that uric acid-induced mitochondria oxidative damage was accompanied by activation of the Rho kinase pathway and that fasudil may contribute to attenuating oxidative stress through suppression of the Rho kinase pathway.

Effects of the Rho kinase pathway on mitochondria-related protein expression in high uric acid-induced HK-2 cells

Next, we investigated the effect of high uric acid levels on mitochondrial-related protein expression. mRNA expression of mitochondrial dyneins (Drp1, Mfn1) and biogenic genes (PGC-1α, NRF-1) was increased in uric acid-induced HK-2 cells compared with the normal control (Figure 3). Fasudil and ROCK1 siRNA significantly reduced Drp1, Mfn1, PGC-1α, and NRF-1 expression, while there was no difference in this gene expression between HK-2 cells that were treated with uric acid and those transfected with sc-siRNA. Therefore, Rho kinase participates in the tubular mitochondrial oxidative injury via regulating mitochondrial

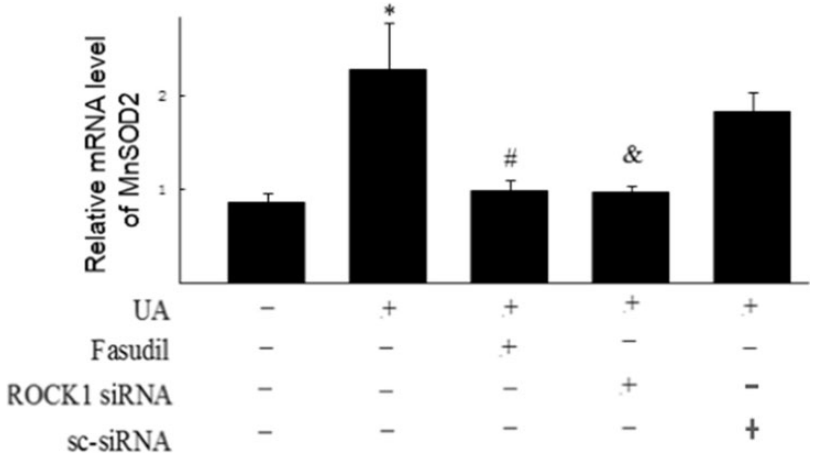

Figure 2. Effects of Rho kinase pathway on mitochondria-induced oxidative damage that was caused by high uric acid in HK-2 cells. HK-2 cells were pre-treated with 10 μmol/L fasudil for 30 minutes, then cultured with 400 μM uric acid for 48 hours. HK-2 cells were transfected with ROCK1 siRNA and sc-siRNA, then cultured with 400 μM uric acid. Real-time PCR analysis showed that high uric acid treatment up-regulated the transcriptions of MnSOD2, while treatment with fasudil and ROCK1 siRNA significantly attenuated high uric acid-induced MnSOD2 mRNA expression. *, $P<0.01$ vs. normal control group; #, $P<0.05$ vs. 400 μM uric acid group; &, $P<0.05$ vs. 400 μM uric acid group.
sc-siRNA, scrambled silencing RNA; PCR, polymerase chain reaction; MnSOD2, manganese superoxide dismutase.

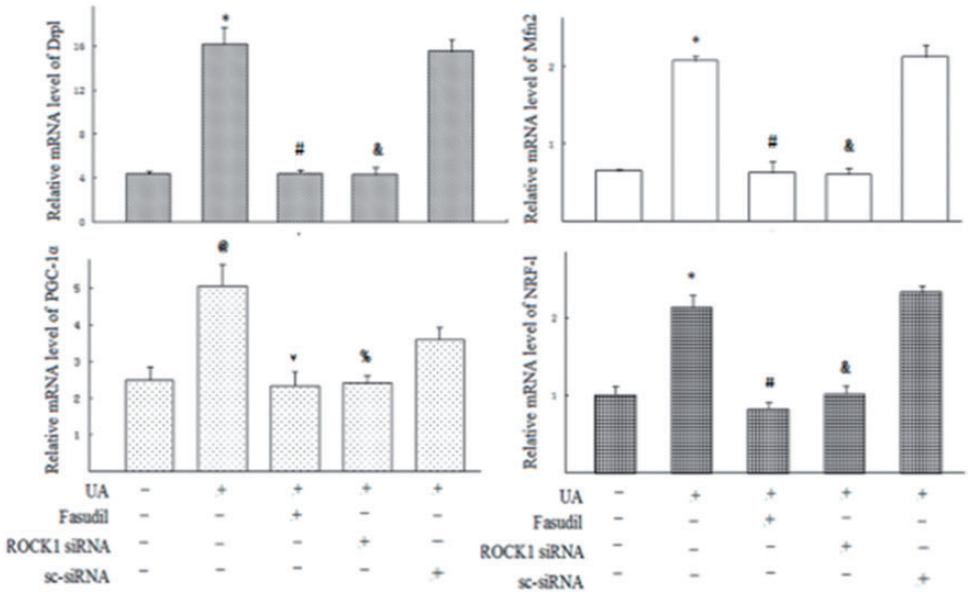

Figure 3. mRNA expression of mitochondria-related proteins in high uric acid-induced HK-2 cells. HK-2 cells were pre-treated with 10 μmol/L fasudil for 30 minutes, then cultured with 400 μM uric acid for 48 hours. HK-2 cells were transfected with ROCK1 siRNA and sc-siRNA, then cultured with 400 μM uric acid. Real-time PCR analysis showed that high uric acid treatment up-regulated Drp1, Mfn1, PGC-1α, and NRF-1 mRNA expression, while treatment with fasudil and ROCK1 siRNA significantly attenuated high uric acid-induced Drp1, Mfn1, PGC-1α, and NRF-1 mRNA expression. *, $P<0.001$ vs. normal control group; #, $P<0.001$ vs. 400 μM uric acid group; &, $P<0.001$ vs. 400 μM uric acid group. @, $P<0.01$ vs. normal control group; ¥, $P<0.01$ vs. 400 μM uric acid group; %, $P<0.01$ vs. 400 μM uric acid group.
sc-siRNA, scrambled silencing RNA; PCR, polymerase chain reaction; Drp1, dynamin-related protein 1; Mfn1, mitochondria for degradation and mitofusin 1; PGC-1α, peroxisome proliferator activated receptor gamma coactivator 1 alpha; NRF-1, nuclear respiratory factor-1.

dyneins and biogenic genes in uric acid-induced HK-2 cells. Similar results were obtained by western blot (Figure 4).

Effects of the Rho kinase pathway on tubular mitochondria apoptosis in high uric acid-induced HK-2 cells

To understand the mechanism of uric acid-induced tubular mitochondrial apoptosis, we examined changes in the expression of pro-caspase-3 and -9, which are the main apoptosis-related proteins. As shown in Figure 2, uric acid increased pro-caspase-3 and -9 expression. Both inhibition of Rho kinase and ROCK1 significantly down-regulated pro-caspase-3 and -9 expression that was induced by uric acid, suggesting that Rho kinase signaling was required for uric acid-induced mitochondrial apoptosis.

Discussion

Our study demonstrated that the Rho kinase signaling pathway was active in high uric acid-induced renal tubular epithelial cells. The Rho kinase inhibitor fasudil and ROCK1 siRNA attenuated the increased MnSOD2, pro-caspase-3, and pro-caspase-9 expression in HK-2 cells that were cultured with high levels of uric acid. The Rho kinase pathway was involved in mitochondrial

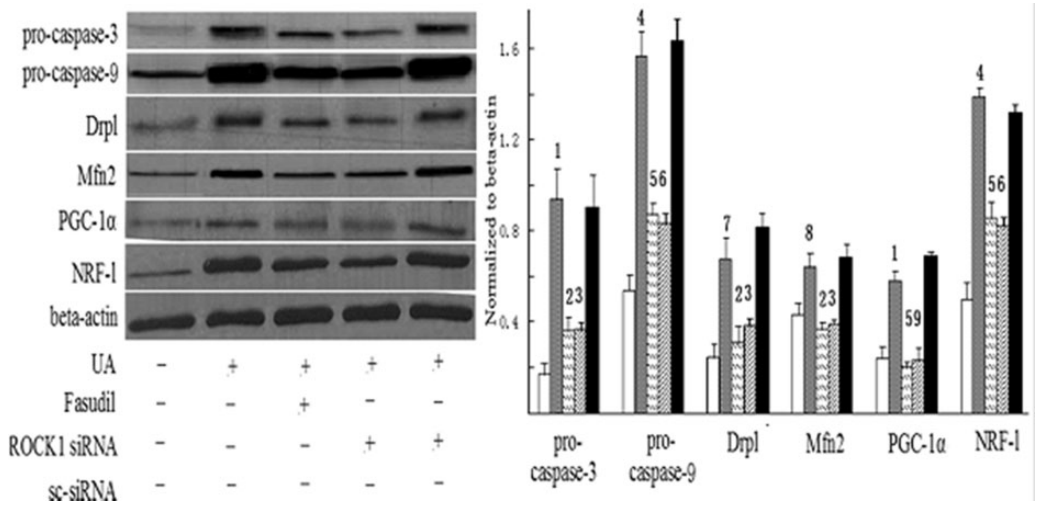

Figure 4. Mitochondria-related protein levels in high uric acid-induced HK-2 cells. HK-2 cells were pre-treated with 10 μmol/L fasudil for 30 minutes, then cultured with 400 μM uric acid for 48 hours. HK-2 cells were transfected with ROCK1 siRNA and sc-siRNA, then cultured with 400 μM uric acid. Western blots showed that high uric acid treatment up-regulated Drp1, Mfn1, PGC-1α, NRF-1, pro-caspase-3, and pro-caspase-9 protein expression while treatment with fasudil and ROCK1 siRNA significantly attenuated high uric acid-induced Drp1, Mfn1, PGC-1α, NRF-1, pro-caspase-3, and pro-caspase-9 protein expression. 1, $P=0.001$ vs. normal control group; 2, $P<0.01$ vs. 400 μM uric acid group; 3, $P<0.01$ vs. 400 μM uric acid group; 4, $P<0.001$ vs. normal control group; 5, $P<0.001$ vs. 400 μM uric acid group; 6, $P<0.001$ vs. 400 μM uric acid group; 7, $P<0.01$ vs. normal control group; 8, $P<0.05$ vs. normal control group; 9, $P=0.001$ vs. 400 μM uric acid group.
sc-siRNA, scrambled silencing RNA; Drp1, dynamin-related protein 1; Mfn1, mitochondria for degradation and mitofusin 1; PGC-1α, peroxisome proliferator activated receptor gamma coactivator 1 alpha; NRF-1, nuclear respiratory factor-1.

oxidative damage and apoptosis of high uric acid-induced renal tubular epithelial cells by regulating Drp1, Mfn1, PGC-1α, and NRF-1 expression.

Previous research has shown that several important intracellular signaling pathways are activated under high uric acid conditions including the TGF-β, ERK1/2, NF-κB, Wnt, and Notch pathways, which play roles in the inflammation response, renin–angiotensin system (RAS) activation, oxidative stress, and tubular epithelial cell transition.[21–24] High uric acid-induced oxidative stress in renal tubular epithelial cells was associated with the overproduction of mitochondrial ROS. MnSOD2 reflects antioxidant enzyme activity, and it can protect cells from oxidative damage by clearing oxygenic radical.[25] Our study demonstrated that high uric acid levels increase MnSOD2 transcription, while fasudil and ROCK1 siRNA down-regulate MnSOD2 mRNA expression. However, Hong et al.[26] noted that high uric acid decreases total superoxide dismutase (T-SOD) activity. These results indicate that inhibition of Rho kinase attenuates mitochondrial oxidative damage by reducing MnSOD2 mRNA expression. Future studies are needed to examine the MnSOD2 activity in HK-2 cells that are exposed to high uric acid levels.

ROS causes mitochondrial dysfunction, ATP synthesis deficiency, and subsequent cell apoptosis.[27] Excessive ROS could

cause destruction of mitochondrial permeability and release of mitochondrial cytochrome C,[28] which activates caspase-3 and -9 to induce apoptosis.[29] Yang et al.[30] confirmed that the higher the uric acid concentration, the is higher the apoptosis rate in renal tubular epithelial cells. Moreover, Yang et al.[31] reported that the expression of apoptotic proteins, such as caspase-3 and -9 in the mitochondria was upregulated in the high uric acid group. We also found that the expression of procaspase-3 and pro-caspase-9, which is the index for pro-apoptotic processes *in vitro*, were increased in the renal tubular epithelial cells after exposure to a high uric acid concentration, and this increase was inhibited by fasudil and ROCK1 siRNA. ROCK1 is the substrate for caspase-3, and it is activated via deleting the C-terminal to regulate the cytoskeleton for apoptosis. The important role of the Rho kinase pathway in tubular mitochondrial apoptosis in high uric acid-induced HK-2 cells was confirmed.

The balance between mitochondrial fusion and fission maintains mitochondrial stability. Individually, Drp1 and Mfn1 are important regulators of mitochondrial fission and fusion. Drp1 mRNA expression is increased in the human kidney, and it may play an important role in the pathophysiology of mitochondrial-targeted injury in the kidney.[32] Previous investigation indicated that suppression of Drp1 accumulation in the mitochondria was favorable for the maintenance of mitochondrial function, and it down-regulated renal tubular cell apoptosis.[33] In our study, suppression of Drp1 by fasudil or ROCK1 siRNA blocked high uric acid-induced mitochondrial fission, mitochondrial dysfunction, and cell injury. Mfn1 as a protein against Drp-1 that participates in the process of mitochondrial fusion. Reducing Mfn1 expression would cause the mitochondrial respiration chain complex activity to decrease and ROS levels to increase, leading to cellular apoptosis.[34] However, our study showed that a high uric acid level upregulates Mfn1 mRNA and protein levels. This result indicates that Mfn1 may promote apoptosis by other pathways. Swiader et al.[35] demonstrated that over-expression of Mnf2 triggers apoptosis though the Ras Raf MEK-ERK/MAPK pathway, which might reduce Bcl-2 expression, increase Bax expression, activate caspase-9, and lead to mitochondrial apoptosis. In addition, Mfn1 is regulated by PGC-1α and NRF-1.[36–38] The data presented in this study suggest that a high uric acid level upregulates PGC-1α and NRF-1 expression, but the Rho kinase inhibitor reversed this response to decrease Mfn1 expression. Taken together, Rho kinase regulates high uric acid-induced mitochondrial oxidative injury and apoptosis via modulation of the balance between fusion and fission in the mitochondria of renal tubular epithelial cells.

In this study, there was also a limitation. An effect of the Rho pathway on mitochondrial-induced oxidative damage should be confirmed using more signaling factors. Rho kinase participates in tubular mitochondrial oxidative injury by regulating mitochondrial dyneins, and the role of biogenic genes should also be confirmed in the future.

Conclusion

The Rho-kinase signaling pathway participates in tubular mitochondrial oxidative injury and apoptosis by regulating mitochondrial dyneins/biogenic genes in uric acid nephropathy. Thus, the mitochondria pathway may represent a potential therapeutic target for hyperuricemia nephropathy.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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