Mitochondrial oxidative damage and apoptosis induced by high glucose through Rho kinase signal pathway in renal tubular epithelial cells

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Objective: To investigate the role of oxidative stress in human renal tubular epithelial cells (HK-2) induced by high glucose and the underlying signal pathway in vitro.

Methods: MYPT1, pro-caspase-3, PGC-1α, and Drp1 protein expressions were measured by Western blot. MnSOD2, Drp1 and PGC-1α mRNA expressions were detected by real time PCR.

Results: Results showed that high glucose significantly up-regulated the protein expressions of MYPT1, pro-caspase-3 and the mRNA expression of MnSOD2 in HK-2 cells; while Rho kinase inhibitor fasudil and ROCK1 siRNA inhibited protein expressions of pro-caspase-3 and the mRNA expression of MnSOD2 in HK-2 cells induced by high glucose. Importantly, fasudil and ROCK1 siRNA markedly inhibited the expressions of mitochondrial motor proteins Drp1 and mitochondrial gene PGC-1α in HK-2 cells induced by high glucose.

Conclusions: Our findings suggest that Rho kinase signal pathway is involved in mitochondrial oxidative damage and apoptosis in high glucose-induced renal tubular epithelial cells by regulating mitochondrial motor proteins Drp1 and mitochondrial gene PGC-1α. Targeting Rho kinase signal pathway might be a potential strategy for the treatment of diabetic nephropathy.

1. Introduction

Diabetic nephropathy (DN) is the major cause of chronic kidney disease and also the primary reason of undergoing kidney replacement therapy[1]. Therefore, to establish a new treatment strategy for DN is of great importance to improve the prognosis of these patients and reduce the socioeconomic burden. Importantly, it is crucial to understand the molecular mechanism of DN.

Over the past decade, a large quantity of evidence have demonstrated that oxidative stress is induced under diabetic conditions[2]. Oxidative stress refers to the overproduction and/or depletion of active molecules, such as reactive oxygen species (ROS) and reactive nitrogen species, resulting in an imbalance between the generation of ROS and antioxidant defenses[3]. Data show that high glucose-induced oxidative stress, particularly the overproduction of mitochondrial ROS in renal tubular epithelial cells, plays a key role in the pathogenesis of DN tubulointerstitial fibrosis. On the one hand, ROS can activate the signal transduction cascade effect, such as PKC, MAPK, JAK/STAT, and activate some transcription factors including NF-κB, ET-1, TGF-β and ECM.
Moreover, these signaling molecules in turn induce ROS production, continually amplifying cellular damage caused by high glucose, eventually leading to progressive glomerulosclerosis and tubular atrophy, and finally causing renal function decline and failure[4].

As an effector of small G protein Rho, Rho kinase has many important physiological and pathological functions, containing cytoskeletal reorganization, cell migration, apoptosis and gene expression[5]. Fasudil, targeting the ATP-dependent kinase domain and inhibiting ROCK1 and ROCK2 equivalently, is the most commonly used pharmacological ROCK inhibitor (ROCKi). Fasudil presents a promising approach to the prevention of breast cancer metastasis, heart failure and DN [6-8]. More researches indicated the hypothesis that ROCKi might be nephroprotective in DN. Kikuchi et al[9] has reported that treatment with fasudil prevents the development of diabetes partially by improving adipocyte differentiation in insulin-resistant diabetic rats. It also has beneficial effects on the glomerulosclerosis and interstitial fibrosis by reducing prosclerotic cytokines and ECM expression, and inhibiting epithelial–mesenchymal transition phenotype[10].

At present, the aim of the study was to investigate the potential mechanisms underlying the role of mitochondrial oxidative stress in the pathogenesis of DN. We hypothesized that inhibited Rho kinase could prevent mitochondrial oxidative stress by modulating the Rho kinase pathway in vivo and in vitro.

2. Materials and methods

2.1. Reagents and antibodies

Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), including rabbit anti-human pro-caspase 3, dynamin-related protein 1 (Drp1), peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1α), myosin phosphatase target subunit 1 (MYPT1) COL1 polyclonal antibodies.

2.2. Cell culture

The immortalized human renal proximal tubular epithelial (HK-2) cell line were purchased from cell bank of Xiangya Central Experiment Laboratory and maintained at 37 °C in an atmosphere of 95% air and 5% CO2 in RPMI-1640, containing 15% FBS, 1% P/S and 1% Glutamax. After digestion with 0.25% trypsin and 0.02% EDTA, 5×10^5 cells were seeded in six-well plates. Subsequently, the cells were treated with the following protocol: (1) HK-2 cells were treated with 5 mM glucose, 30 mM glucose and 30 mM mannitol for 48 h; (2) HK-2 cells were pre-treated with 10 μmol/L fasudil for 30 min and then cultured with 30 mM glucose for 48 h; (3) HK-2 cells were transfected with ROCK1 siRNA or sc-siRNA before treated with 30 mM glucose.

2.3. Real-time PCR

Total RNA of HK-2 cells were extracted using Trizol Total RNA Isolation kit (Invitrogen). Reverse transcription was performed using the Superscript III RT kit (Invitrogen) according to the manufacturer’s instruction. Briefly, the primer sequences designed by Primer 5 software were synthesised at the Invitrogen Corporation (Shanghai, China) and listed in Table 1. The reactions were incubated as follows: 94 °C for 5 min, 94 °C 30 s, 59 °C 30 s, 72 °C 30 s, 35 cycles, 72 °C for 5 min.

| Table 1 |
| Primers used in this study. |
| Primers | Sequence |
| Drp1 (198 bp) | 5’-GGGTTGAGATGGTG-3’ |
| PGC-1α (173 bp) | 5’-GAATAGTGCGTGCC-3’ |
| MnSOD2 (197 bp) | 5’-GGGTTGGCTTGGTTC-3’ |
| β–actin (231 bp) | 5’-ACTCTTCCAGCCTTCCTTCC-3’ |

2.4. Western blot analysis

HK-2 cells were lysed in 2×SDS–PAGE sample buffer. Total protein concentrations were measured with a BCA kit (Applygen). Lysate proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Then, the membranes were incubated with the following primary antibodies overnight at 4 °C: anti-pro-caspase 3, anti-Drp1, anti-PGC-1α, anti-MYPT1, anti-COL1, beta-actin. Subsequently, the membranes were washed with PBS-Tween and incubated with secondary antibodies for 2 h at room temperature. Immunoreactivity was scanned using an ECL system (Amersham) and the Bio-Rad Electrophoresis Image Analyzer (Bio-Rad, Hemel Hampstead, UK).

2.5. Statistical analysis

Data were expressed as the means ± standard deviation and analyzed using SPSS 20.0 for Windows (SPSS, Chicago, IL, USA). Data were calculated for at least three independent experiments. For comparisons of different groups, Student’s t-test was used, P < 0.05 was considered as statistically significance.

3. Results

3.1. Rho kinase participated in high glucose-induced HK–2 cells
To explore the activity of Rho kinase in high glucose-induced HK-2 cells, we measured the level of MYPT1 which was one of the major substrates of ROCK. In high glucose group, the MYPT1 protein levels were significantly higher than those of 5 mM glucose group and mannitol group (Figure 1).

The Rho kinase signal pathway was involved in high glucose-induced mitochondrial-mediated oxidative damage and apoptosis in HK-2 cells. HK-2 cells were incubated with 5 mM glucose, 30 mM glucose and 30 mM mannitol for 48 h (n=3). Western blots showed that treatment with 30 mM glucose significantly increase the expression of MYPT1 protein. *: P<0.01 vs. 5 mM glucose control group; #: P = 0.01 vs. 30 mM glucose group.

### 3.2. Effects of Rho kinase pathway on mitochondria-induced oxidative damage and apoptosis induced by high glucose in HK-2 cells

HK-2 cells were treated with fasudil at a dose of 10 μM, and ROCK1 siRNAs were successfully transfected into HK-2 cells and then these cells were cultured with 30 mM glucose, separately. Administration of high glucose for HK-2 cells up-regulated the transcription of manganese superoxide dismutase (MnSOD2) by real-time PCR. Further, treatment with fasudil and ROCK1 siRNA significantly suppressed the expression of high glucose-induced MnSOD2 mRNA expression, respectively (Figure 2). These data suggested that Rho kinase pathway participated in mitochondria-induced oxidative damage induced by high glucose in HK-2 cells.

In addition, our results showed that high glucose treatment up-regulated the expression of pro-caspase-3 protein expression. Treatment with fasudil and ROCK1 siRNA significantly antagonized high glucose-induced pro-caspase-3 protein expression, respectively (Figure 3). These data indicated that Rho kinase pathway was involved in mitochondria-related apoptosis induced by high glucose in HK-2 cells.

### 3.3. Effects of Rho kinase pathway on Drp1 and PGC-1α expression in high glucose–induced HK-2 cells

Mitochondrial morphological changes are controlled by a series of dynamin family GTP-binding proteins, including Drp1. PGC-1α is a regulator of mitochondrial biosynthesis and participates in oxidative phosphorylation. As shown in Figure 4, we measured the expression of Drp1 and PGC-1α in order to explore the mechanism of Rho kinase pathway in high glucose-induced HK-2 cells. In high glucose group, Drp1 and PGC-1α were up-regulated compared with the control group. Importantly, fasudil and ROCK1 siRNA markedly suppressed the expression of Drp1 and PGC-1α compared with high glucose group. Our results suggested that Rho kinase pathway regulated the mitochondrial oxidative damage of high glucose-

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**Figure 1.** Upregulation of MYPT1 protein in HK-2 cells induced by high glucose.

**Figure 2.** Relative mRNA level of MnSOD2.

HK-2 cells were pre-treated with 10 μmol/ L fasudil for 30 min and then cultured with 30 mM glucose for 48 h. HK-2 cells were transfected with ROCK1 siRNA and sc-siRNA, and then cultured with 30 mM glucose. Real-time PCR analysis showed that high glucose treatment up-regulated the transcriptions of MnSOD2, while treatment with fasudil and ROCK1 siRNA significantly attenuated high glucose-induced MnSOD2 mRNA expression. *: P<0.001 vs. 5 mM glucose group; #: P<0.05 vs. 30 mM glucose group; &: P<0.001 vs. 30 mM glucose group.

**Figure 3.** Relative protein level of pro-caspase-3.

Western blots showed that high glucose up-regulated the expression of pro-caspase-3 protein expression, while treatment with fasudil and ROCK1 siRNA significantly antagonized high glucose-induced pro-caspase-3 protein expression. n=3 for each group. *: P<0.01 vs. 5 mM glucose group; #: P = 0.01 vs. 30 mM glucose group; &: P<0.05 vs. 30 mM glucose group.
Our study demonstrated that Rho kinase signaling pathway was involved in the high glucose-induced renal tubular epithelial cell. The Rho kinase inhibitor fasudil and ROCK1 siRNA attenuated the increased levels of MnSOD2 and pro-caspase-3 expression levels in HK-2 cells cultured in high glucose. Rho kinase pathway was involved in mitochondrial oxidative damage and apoptosis of high glucose-reduced renal tubular epithelial cells by regulating Drp1 and PGC-1α expression.

According to previous research, a number of important intracellular signaling pathways were activated in high glucose conditions including the TGF-β/SMAD, nuclear factor-κB and Rho/ROCK[11-13]. They played roles in the inflammation response, RAS activation, epithelial–mesenchymal transition and fibrosis of DN[14,15]. However, we focused on the oxidative damage, in which Rho kinase signaling pathway may serve crucial roles. The production of ROS is closely related to the high glucose-induced oxidative stress in renal tubular epithelium. Most of intracellular ROS comes from mitochondria. Reactive oxygen can cause lipid peroxidation in the mitochondrial membrane, resulting in impaired mitochondrial function, insufficient ATP synthesis, increased free radicals, loss of energy in the cells, increased intracellular calcium concentration, and ultimately lead to apoptosis[16,17]. In this study, we used ROCKi and ROCK1 siRNA to inhibit the activation of Rho kinase pathway. Fasudil competes with ATP for the ATP binding site in the ROCK catalytic domain and blocks ROCK activity through competitive antagonism, thereby blocking the ROCK-mediated biological function[9]. The superoxide dismutase family is a key enzyme in scavenging oxygen free radicals. Among them, MnSOD2 plays a major role, and it is also a marker of oxidative stress[18]. MnSOD2 was significantly up-regulated in high glucose

induced HK-2 cells by regulating the expression of mitochondrial motor protein Drp1 and mitochondrial gene PGC–1α.

4. Discussion

Our study demonstrated that Rho kinase signaling pathway was involved in the high glucose-induced renal tubular epithelial cell. The Rho kinase inhibitor fasudil and ROCK1 siRNA attenuated the increased levels of MnSOD2 and pro-caspase-3 expression levels in HK-2 cells cultured in high glucose. Rho kinase pathway was involved in mitochondrial oxidative damage and apoptosis of high glucose-reduced renal tubular epithelial cells by regulating Drp1 and PGC-1α expression.

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Figure 4. Mitochondria related proteins levels in high glucose-induced HK-2 cells.

(A, B) High glucose up-regulated the mRNA expressions of Drp1 and PGC–1α. Fasudil and ROCK1 siRNA pretreatment significantly antagonized high glucose induced Drp1 and PGC–1α mRNA transcriptions. *: \( P<0.05 \) vs. 5 mM glucose group; #: \( P<0.05 \) vs. 30 mM glucose group; (C) High glucose increased the expression of Drp1 protein, and fasudil and ROCK1 siRNA pretreatment significantly antagonized high glucose-induced Drp1 protein expression. "\( P<0.01 \) vs. 5 mM glucose group; ##: \( P=0.001 \) vs. 30 mM glucose group; (D) High glucose up-regulated the expression of PGC–1α protein, and fasudil and ROCK1 siRNA pretreatment significantly antagonized high glucose-induced PGC–1α expression. @: \( P=0.01 \) vs. 5 mM glucose group; $: \( P<0.01 \) vs. 30 mM glucose group.
group. More importantly, fasudil and ROCK1 siRNA attenuated the high glucose-induced MnSOD2 mRNA expression, indicating that oxidative stress was inhibited, and mitochondrial oxidative damage to renal tubular epithelial cells was reduced.

Glomerular lesions was traditionally associated with DN. However, evidence has increasingly shown that renal tubule also played a crucial role in DN in recent years[19,20]. Our results are consistent with those results that high glucose-induced oxidative stress and apoptosis are increased in DN[21]. Meanwhile, we observed that the expression of pro-caspase-3, an index for pro-apoptotic processes in vitro, was up-regulated in the renal tubular epithelial cells after exposure to high glucose environment. Excessive ROS could cause destruction of mitochondrial permeability and release of mitochondrial cytochrome C[22], which activated caspase-3 and -9 to induce apoptosis[23]. Moreover, increasing evidence support the role of Rho Rho kinase pathway in DN[24]. Our data show that interference of the ROCK1 expression and Rho kinase activity can reduce high glucose-induced tubular apoptosis. ROCK1 is cleaved by caspase-3 at the C-terminal domain to activate constitutively, and regulate cytoskeleton to induce apoptosis. Taken together, this study demonstrate that Rho kinase pathway may take part in hyperglycemia-induced renal tubular epithelial cell mitochondria-mediated apoptosis.

Mitochondria-targeting antioxidants have been suggested as a possible therapeutic strategy to prevent and treat the the development of DN[25,26]. In addition, as in other cells, the Rho signaling pathway is activated in renal cells of the diabetic milieu environment[27]. Studies have demonstrated that the pathway contributes to progrowth, prosclerotic/profibrotic signalling, and increased ECM production[7,28,29]. Furthermore, it mediates the activation of the kidney myofibroblast and participates in the pathophysiological progression of renal fibrosis[30,31]. Indeed, studies showed that ROCK1 was induced by high glucose and then stimulated the endothelial-to-mesenchymal transition[32]. Our data provides evidence that the Rho kinase pathway regulates oxidative damage and apoptosis of high glucose-induced renal tubular epithelial cells by regulating the expression of Drp1 and PGC-1α.

More importantly, the present study suggests that mitochondrial oxidative stress regulated by Rho kinase pathway plays a key role in renal tubulointerstitial fibrosis in DN. Further studies using gene knockout animal models and more clinical trials using antioxidants will be required to elucidate the distinct roles of mitochondrial oxidative stress in renal tubular epithelial cells in DN.

In summary, our evidence shows that Rho kinase pathway is involved in mitochondrial oxidative stress of high glucose-reduced renal tubular epithelial cells by regulating Drp1 and PGC-1α. This study provides new insights into the pathogenesis of DN and it should help develop a new therapeutic strategy for DN.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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