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Research Article

PGC-1α and P38 MAPK are involved in Uncoupling Protein 2-Dependent Reactive Oxygen Species Production in Renal Tubular Epithelial Cells

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ABSTRACT

Object: The objective was to investigate Uncoupling protein 2 (UCP2)-dependent Reactive oxygen species (ROS) production in renal tubular epithelial cells treated with high glucose.

Methods: In this study, we investigated the associations among PGC-1α, p38 MAPK and UCP2 in HK-2 cells treated with high glucose. Target genes overexpression and silence were obtained by plasmid transfection, and SB203580 was applied as a p38 MAPK inhibitor. The mutual effect of these three proteins was evaluated by RT-PCR and western blot. ROS production was detected by a fluorescent probe.

Results: We found that UCP2 overexpression enhanced PGC-1α expression, as well as inhibited p38 MAPK activation and ROS generation. These changes were reversed by UCP2 siRNA. However, neither PGC-1α nor p38 MAPK could exert significant influence on the expressions of the other two proteins, although their capacity to regulate ROS was demonstrated.

Conclusion: These results indicate UCP2 plays a pivotal and relatively upstream role in oxidative stress induced by high glucose in renal tubular epithelial cells.

Keywords: Uncoupling protein-2; Peroxisome proliferator-activated receptor γ co-activator 1-α; p38 mitogen-activated protein kinase; Reactive oxygen species; Diabetic kidney disease

INTRODUCTION

Diabetic kidney disease (DKD) develops in approximately 40% of patients with diabetic mellitus and is the leading cause of end stage renal disease worldwide. Metabolic changes associated with diabetes lead to glomerular hypertrophy, glomerulosclerosis, and tubulointerstitial inflammation and fibrosis [1]. Numerous pathways and risk factors are involved in the initiation and progression of diabetic kidney disease. Among them, reactive oxygen species (ROS) have been proved to play a pivotal role [2, 3]. Overproduction of ROS in DKD is both a direct consequence of hyperglycemia and an indirect consequence through mediators of glucotoxicity, such as advanced glycation end products, cytokines and growth factors. Nicotinamide adenosine dinucleotide phosphate oxidase and mitochondrial dysfunction have been recognized as two major sources of ROS generation in diabetic kidneys [4].

The respiratory chain in mitochondria serves as a major source of ROS under both physiological and diabetic condition [5]. Thus, downregulation of ROS deriving from mitochondria may be a promising approach for DKD treatment. Uncoupling protein 2 (UCP2), a mitochondrial membrane protein, has been reported to be associated with reduction of mitochondrial ROS generation [6, 7]. In our previous study, we demonstrated that UCP2 exerted a protective effect against oxidative stress and ameliorated apoptosis in renal tubular epithelia cells treated with high glucose [8]. In this study, we further study the mechanism of UCP2 as the core molecule regulating ROS.

Peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α) is a transcriptional coactivator identified as an upstream regulator of mitochondrial biogenesis and oxidative metabolism [9]. Besides, it has been demonstrated that PGC-1α activates transcription of UCP2 mRNA in tissues where mitochondria are abundant, such as skeletal muscle and pancreatic β-cells [10, 11]. However, if PGC-1α is also involved in UCP2 gene expression in diabetic kidney disease remains unclear. Additionally, p38 mitogen-activated protein kinase (MAPK), a member of family of serine/threonine kinases, is an important stress signaling molecule and is involved in many ROS related cellular dysfunctions. Valouskova E et al. [12] considered a tentative relationship between p38 MAPK and

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UCP2 in mitochondrial ROS production. They presumed tissue specific regulation account for the divergent effects. In the present study, we intended to elucidate whether PGC-1α and p38 MAPK participate in the ROS generation modulated by UCP2 in high glucose treated renal tubular epithelial cells. We observed that UCP2 could influence the expression of PGC-1α and p38 MAPK. This regulatory activity helps to prevent ROS production under high glucose conditions.

METHODS

Cells

Human renal tubular epithelial cell lines (human kidney 2, HK-2) were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified eagle's medium/F12 (Hyclone, NY, USA) supplemented with 10 % (v/v) fetal bovine serum (Hyclone, NY, USA). The cultures were maintained in a humidified incubator at 37 °C under 5% CO2. The cells were maintained in serum-free media for 24 h before each treatment. Cells incubated with 5.5 mmol/L glucose served as control group; Cells in high glucose (HG) group were incubated with 30 mmol/L glucose.

Reagents

Genipin (G4796) and β-actin (A5316) antibodies were purchased from Sigma Aldrich (St. Louis, MO, USA). PGC-1α (2178), UCP2 (89326), p38 MAPK (9212), phosphorylated-p38 MAPK (p-p38 MAPK, 9216) antibodies and SB203580 (5633) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Plasmid construction, siRNA and transfection

Full length human PGC-1α and UCP2 were cloned with pcDNA3.1. The siRNA against human UCP2 was chemically synthesized by GenePharma (Shanghai, China) as the following oligonucleotide sequences: sense: 5'-GCACCGUCAAUGCCUACAATT-3', antisense: 5'-UUGUAGGCAUUGACGGUGCTT-3'. Transient transfection of plasmid and siRNA were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). A total of 5 mg of plasmid or 100 pmol of siRNA containing Lipofectamine was applied in a final volume of 0.75 ml per well in 6-well plates.

RNA extraction and RT-PCR analysis

HK-2 cells were seeded into 6-well plates at a density of 2 × 105 cells/well in 2mL medium. Total RNA was isolated using trizol reagent. Oligo (dT)-primed RNA (1 μg) was reverse transcribed with Revert AidM-MuLV reverse transcriptase (Fermentas, Amherst, NY) according to the manufacturer's instructions. The obtained cDNA was used to determine the amount of UCP2 and β-actin mRNA, using PCR with Taq DNA polymerase (TAKARA). β-actin was used as an internal control. The primers that were used for amplification of the UCP2 and β-actin transcripts were as follows: UCP2 forward, 5'-CTCTGGAAAGGGACCTCTCCCA-3' and reverse, 5'-AGGCAGAAGTGAAGTGGCAAGG-3'; β-actin forward, 5'-GAAGATCAAGATCATTGCTCCT-3' and reverse, 5'-TACTCCTGCTTGCTGATCCACA-3'. PCR condition is one cycle of 94 °C for 2 min, 45 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s and one cycle of 72 °C for 5 min. The PCR products, which were fractionated on a 1% agarose gel containing ethidium bromide and visualized under UV light.

Western blot

Western blot were performed as described elsewhere [13, 14]. Briefly, the cells or the membrane fractions were resuspended in lysis buffer containing Nonidet P-40 (10 mM Hepes, pH 7.4, 2 mM EGTA, 0.5% Nonidet P-40, 1 mM NaF, 1 mM NaVO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 50 μg/ml trypsin inhibitor, 10 μg/ml aprotinin, and leupeptin) and placed on ice for 30 min. The lysates were centrifuged at 12,000 × g for 12 min at 4 °C, and the protein concentration was determined. Equivalent samples (30 μg of protein) were subjected to SDS-PAGE on 12% gels. The proteins were then transferred onto nitrocellulose membranes and probed with the indicated antibodies followed by the appropriate secondary antibodies conjugated to horseradish peroxidase (KPL, Gaithersburg, MD). Immunoreactive bands were visualized using enhanced chemiluminescence (Pierce, Rockford, IL, USA). The molecular sizes of the proteins detected were determined by comparison with prestained protein markers (Invitrogen). Densitometric analysis of the Western blots was performed as described before [15].

ROS detection

ROS production was detected using 5-(and-6)-chloromethyl-20,70-dichlorodihydrofluorescein diacetate (CM-H2DCFDA), an uncharged, cell-permeable fluorescent probe (C6827, Invitrogen Molecular Probes, Eugene, OR, USA). When being incubated with the cells, this dye can readily diffuse into cells and is hydrolysed by intracellular esterases to yield H2DCF, which is trapped within the cells. Then, it is oxidized from the nonfluorescent form to a highly fluorescent compound by the hydrogen peroxide or other low-molecular-weight peroxides produced in the cells. Thus, the fluorescence intensity is proportional to the amount of peroxide produced by the cells. Exponentially growing cells (1 × 105 cells/ml) were labeled with 1 mM CM-H2DCFDA for 1 h before treatment, and then were washed with PBS. The green fluorescence intensity in the cells was examined by FACS (Becton Dickinson, San Jose, CA, USA). ROS levels were analyzed by CellQuest software (Becton Dickinson).

Statistical analysis

The statistical analysis was performed with SPSS software (version 17.0 for Windows). Results are presented as mean ± S.D. Analysis of variance and the Tukey-Kramer multiple-comparison test were used in comparisons. $P<0.05$ was considered statistically significant.

RESULTS

UCP2 and PGC-1α increased at the early stage after HG treatment, but decreased later. We observed the protein expression change of UCP2 and PGC-1α for 72 hours in HK-2 cells treated with HG. UCP2 and PGC-1α increased obviously from 12 to 48 hours after HG treatment, while decreased significantly after 72 hours. (Figure 1). These results may reflect a compensatory effect of UCP2 at early stage after HG stimulation. Besides, p38 MAPK was activated right after HG treatment (Figure 1).

Genipin decreased UCP2 and PGC-1α protein expression, meanwhile enhanced p38 MAPK activation and ROS production to investigate the change of PGC-1α and p38 MAPK after UCP2 ablation in the condition of high glucose, we used genipin, a UCP2 inhibitor, to treat HK-2 cells. We found that genipin decreased the protein expression of UCP2 and PGC-1α in both HG group and control group (Figure 2a). However, phosphorylated p38 MAPK was enhanced by genipin (Figure 2a), which was accompanied by more ROS production (Figure 2b). These results suggest that UCP2 is implicated in the regulation of PGC-1α expression and p38 MAPK activation in the oxidative stress caused by HG.

UCP2 enhanced PGC-1α expression, and inhibited p38 MAPK activation and ROS production. We then determined the role of UCP2 in regulation of PGC-1α and p38 MAPK. Constitutively active UCP2 (encoded by plasmid) and UCP2-specific siRNA were introduced to HK-2 cells. We found that UCP2 overexpression increased the expression of PGC-1α and decreased p-p38 MAPK (Figure 3a), which were coupled by dramatically decreased ROS levels (Figure 3b). In contrast, the inhibition of UCP2 by siRNA further decreased PGC-1α expression and promoted p-p38 MAPK (Figure 3c). Moreover, UCP2 inhibition increased ROS levels (Figure 3d). These results suggest that UCP2 indeed promotes PGC-1α expression, reduces p38 MAPK activation and inhibits ROS production in the presence of HG.

PGC-1α only increased UCP2 mRNA expression and had no effect on p38 MAPK activation HK-2 cells were transfected with the PGC-1α expression plasmid to demonstrate whether PGC-1α has the capacity to regulate UCP2 expression, p38 MAPK activation and ROS production in the presence of HG. We found that PGC-1α overexpression had little effect on protein expression of UCP2 and p38 MAPK activation in the

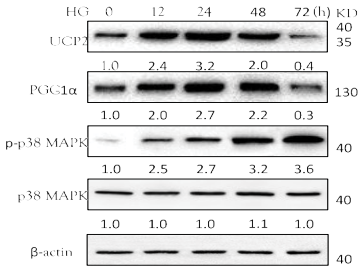

Figure 1: High glucose (HG) induces p38 MAPK and ROS activation in HK-2 cells. HK-2 Cells were treated with HG (30 mM) for different periods of time, and then collected for detection of indicated protein expression. β-Actin was used as a protein loading control. Densitometric analysis of the Western blots was performed and the amount of UCP2, PGC-1α, p-p38 MAPK or p38 MAPK was compared to the protein loading control as described before [15]. The relative amount from untreated cells (0 h) was set at 1 for UCP2, PGC-1α, p-p38 MAPK or p38 MAPK. All data are representative of three independent experiments.

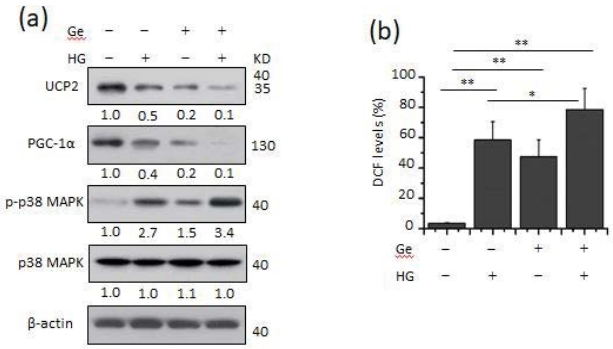

Figure 2: Genipin (Ge) increases p38 MAPK activation and ROS production, but decreases UCP2 and PGC-1α expression in HG-treated renal tubular cell line. (a) Cells were treated with HG and/or Ge (40 μM), added to the cell cultures 30 min prior to changing the medium to a high glucose medium) for 72 h, and then collected for detection of indicated protein expression. β-Actin was used as a protein loading control. Densitometric analysis of the Western blots was performed as Figure 1. Relative amount of individual protein from untreated cells (0 h) was set as 1. (b) Cells were treated as described in (a), and then ROS levels were detected. Each histogram shows the levels of ROS in treated cells (n=3, mean ± S.D. *, P<0.05; **, P<0.01). Representative results of three western blot experiments with consistent results are shown.

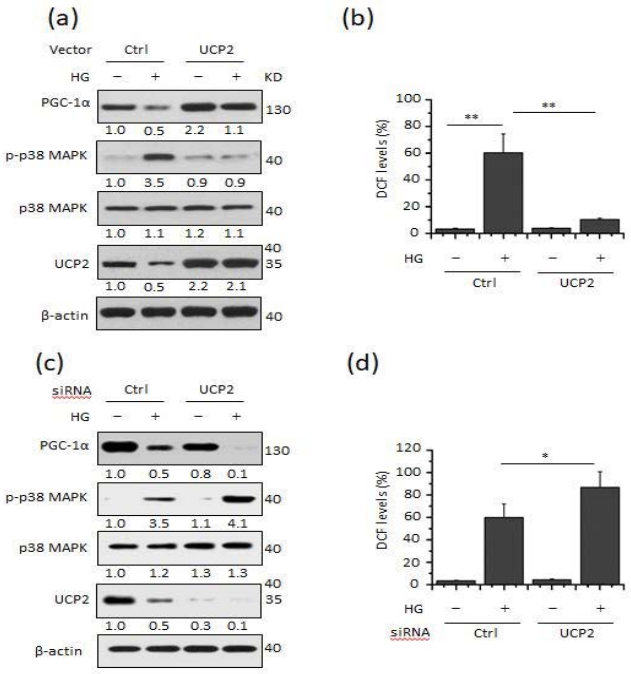

Figure 3: UCP2 regulates PGC-1α expression, p38 MAPK activation and ROS production in HG-treated renal tubular cell line. (a) Cells were transfected with ctrl or UCP2 vector for 48 h, and then cells were treated with HG for 72 h. Treated cells were collected for detection of indicated protein expression. β-Actin was used as a protein loading control. Densitometric analysis of the Western blots was performed as Figure 1. Relative amount of individual protein from untreated cells (0 h) was set as 1. (b) Cells were treated as described in (a), and then ROS levels were detected. Each histogram shows the levels of ROS in treated cells (n=3, mean ± S.D. **, P<0.01). (c) Cells were transfected with ctrl or UCP2 siRNA for 48 h, and then cells were treated with HG for 72 h. Treated cells were collected for detection of indicated protein expression. Densitometric analysis of the Western blots was performed as Figure 1. Relative amount of individual protein from untreated cells (0 h) was set as 1. (d) Cells were treated as described in (c), and then ROS levels were detected. Each histogram shows the levels of ROS in treated cells (n=3, mean ± S.D. *, P<0.05). All data are representative of three independent western blot experiments.

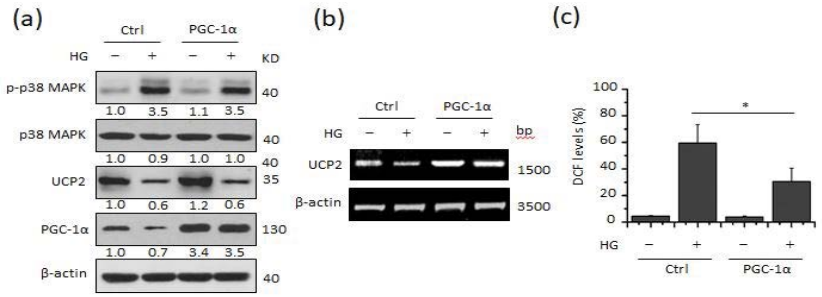

Figure 4: PGC-1α expression has little effect on UCP2 protein expression and p38 MAPK activation in the presence of HG. (a) Cells were transfected with ctrl or PGC-1α vector for 48 h, and then cells were treated with HG for 72 h. Treated cells were collected for detection of indicated protein expression. β-Actin was used as a protein loading control. Densitometric analysis of the Western blots was performed as Figure 1. Relative amount of individual protein from untreated cells (0 h) was set as 1. (b) Cells were transfected with ctrl or PGC-1α vector for 48 h, and then cells were treated with HG for 72 h. Treated cells were collected for RT-PCR. (c) Cells were treated as described in (a), and then ROS levels were detected. Each histogram shows the levels of ROS in treated cells (n=3, mean ± S.D. *, P<0.05). Representative results of three western blot experiments with consistent results are shown.

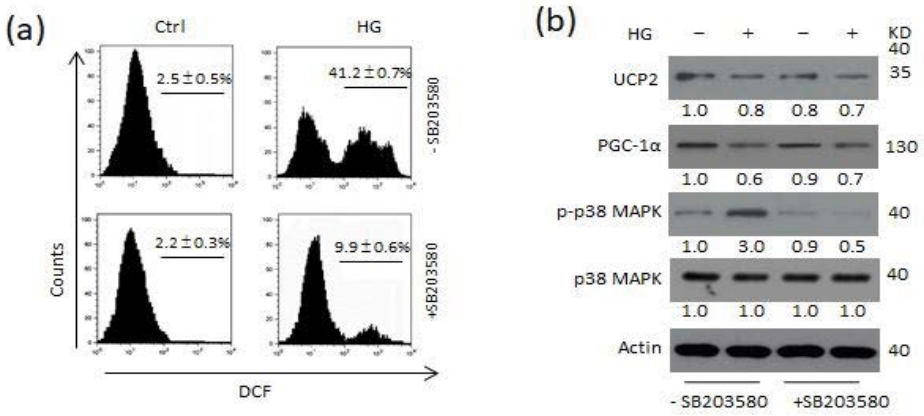

Figure 5: p38 MAPK contributes to ROS production, but have no influence on PGC-1α and UCP2 expression in the presence of HG. (a) Cells were treated with HG and SB203580 (30 μM) for 48 h. Untreated and treated cells were then washed in PBS and incubated with 20 μM H2DCFDA at 37°C for 30 min. DCF fluorescence (ROS levels) were detected using a FACStar flow cytometer. (b) Cells were treated with HG and SB203580 (30 μM) or HG alone for 72h, then collected for detection of indicated protein expression. β-Actin was used as a protein loading control. Densitometric analysis of the Western blots was performed as Figure 1. Relative amount of individual protein from untreated cells (0 h) was set as 1. All data are representative of three independent western blot experiments.

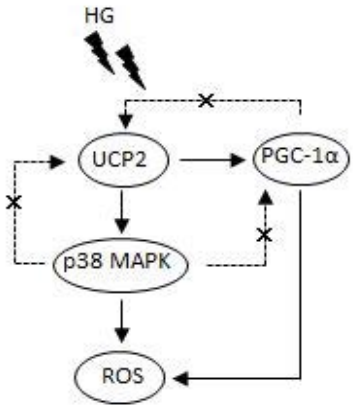

Figure 6: A diagram of signaling pathway for UCP2-dependent reactive oxygen species production in renal tubular epithelial cells. HG treatment reduced UCP2 and PGC-1α exrepssion. Meanwhile, HG treatment activated p38 MAPK and induced ROS production. UCP2 is the upstream regulator of the activity of PGC-1α, p38 MAPK and ROS.

presence of HG (Figure 4a), although UCP2 mRNA expression were enhanced (Figure 4b). In addition, significantly reduced ROS production was observed, albeit the extent of which is smaller than that induced by UCP2 overexpression (Figure 4c).

P38 MAPK contributed to ROS production, but had no influence on PGC-1α and UCP2 in HG-treated cells to establish the role of p38 MAPK in oxidative stress induced by high glucose, we applied SB203580, a p38 MAPK inhibitor, to HK-2 cells. SB203580 decreased ROS levels marked with DCF fluorescence in cells (Figure 5a), indicating that p38 MAPK activation contributes to ROS production in HG condition. However, there were no detectable effects of SB203580 on the expressions of PGC-1α and UCP2 (Figure 5b). These data suggest that p38 MAPK is not an upstream regulator of PGC-1α and UCP2 expression but contributes to ROS production in HG condition.

DISCUSSION

Oxidative stress has been considered as a key component in the development of diabetic nephropathy. Especially, the generation of ROS by damaged or dysfunctional mitochondria is postulated as the primary initiating event in the development of diabetes complications [16]. Therefore, suppressing mitochondrial-derived ROS may serve as a novel treatment for DKD. The protective role of UCP2 in oxidative stress damage resulted by certain inflammatory diseases has been identified, such as diabetic retinopathy and hypertension [17-19]. Our previous study showed that UCP2 could be important in the prevention of oxidative stress damage in renal tubular epithelial cells induced by hyperglycemia in vitro [8]. In this study, we further elaborated the mechanisms by which UCP2 regulates ROS production under high glucose treatment. Firstly, we detected that the protein expression of UCP2 and PGC-1α increased gradually from 12 to 48 hours after HG treatment, while decreased significantly after 72 hours. These results suggest that UCP2 and PGC-1α increase in a compensatory manner at the early stage of high glucose stimulation, which are consistent with previous study [17, 20-22]. The previous study found that diabetes could lead to increase the PGC-1α-related pathway to handle the excess lipid production in the early states. But in the late stages, excess fatty acid leaded to generation of toxic lipids, accumulation of reactive oxygen species. So the PGC-1α-related was diminished [22].We gets hints from this literature. In our system, we speculated that the increased expression of UCP2 and PGC-1α at the beginning of high glucose treatment can serve as a protective mechanism against injury. However, continuous high glucose treatment perhaps leads to the accumulation of reactive oxygen species in cells. This increase in ROS goes beyond the capacity of the protection mechanism, resulting in the decrease of UCP2 and PGC-1α expression in the late stage. We then found that UCP2 expression could be down-regulated in HK-2 cells incubated with genipin, besides decreased PGC-1α expression and more p38 MAPK activation were induced. Genipin, which is an inhibitor of UCP2, has been verified to promote ROS production [23]. Therefore, we speculated whether UCP2 could regulate ROS production by influencing PGC-1α expression as well as the activity of p38 MAPK. In this study, we did verify that up or down regulation of UCP2 could affect the expression of PGC-1α and the phosphorylation level of p38 MAPK, which were accompanied by ROS level change.

Interestingly, PGC-1α has been shown as an upstream regulator of UCP2. PPAR-γ/PGC-1α expression can reduce mitochondrial membrane potential, ROS production and potential apoptotic events through the induction of UCP2 in central nervous system [24, 25]. Besides, PGC-1α/UCP2 axis is reported to be involved in mitochondrial $O2^{-}$• production in cancer cells [26]. For diabetic kidney disease, recent studies have shown that the mRNA and protein expression of PGC-1α are markedly downregulated in renal tubular cells of streptozotocin-induced diabetic rats [27]. Wu et al. discovered that FOXO3a, which is a direct transcriptional regulator of a group of oxidation-protection genes, can reverse the ROS generation induced by high-glucose in a PGC-1α-dependent mechanism in rat kidney mesangial cells [28]. Therefore, we further investigated the effect of up-regulation of PGC-1α on UCP2 expression. We found that overexpression of PGC-1α reduced ROS production with UCP2 mRNA expression level increasing, while UCP2 protein expression level was not affected in HK-2 cells incubated by high glucose. These results indicated that although PGC-1α is implicated in the gene expression of UCP2 in renal tubular cells under high glucose treatment, the protein expression of it is probably controlled by other factors. PGC-1α may regulate oxidative stress response in diabetic renal tubular injury in a UCP2 independent pathway. The discrepancy with other studies may be attributed to the tissue-specific protein expression.

In addition, we also pay attention to the role of p38 MAPK in high glucose induced oxidative stress. p38 MAPK is a member of the MAPK family, which is known to play a regulatory role in inflammation in various disease, including diabetic mellitus. Suppression of p38 MAPK activity has been shown to be beneficial for renal functions in type 2 diabetic rats [29]. Studies have corroborated that oxidative stress is a key factor for the activation of p38 MAPK. On one hand, excessive ROS production induced by hyperglycemia is capable of activating p38 pathway, which controls the expression of various genes associated with inflammation. On the other hand, once p38 MAPK pathway is activated, it can damage antioxidative system, thereby in turn further increasing intracellular ROS level. That means ROS and p38 MAPK exhibit a mutual regulation in oxidative stress [30]. In our study, SB203580, a p38 MAPK inhibitor, decreased ROS levels induced by HG. This result indicates that p38 MAPK activation contributes to ROS production, which is consistent with previous studies [31-33]. Moreover, p38 MAPK has been demonstrated to be involved in modulation of UCP2 and PGC-1α expression [12, 34]. However, our results did not show p38 MAPK inhibitor's influence on both UCP2 and PGC-1α expression. On contrary, we detected that UCP2 was implicated in p38 MAPK phosphorylation. Functional knockdown of UCP2 by small interfering RNA-mediated silencing was associated with increased p38 MAPK activity. This result is also identified by other researchers [35]. It is plausible to attribute this effect partly to the up-regulation of ROS resulted from UCP2 ablation, which is necessary to potentiate MAPK activation. Basu Ball W et al. also found p38 MAPK could be activated in UCP2 knocked-down cells, which was considered to result from ROS-mediated inhibition of protein tyrosine phosphatases (PTPs) [36]. Whether UCP2 exerts a direct regulation on p38 MAPK activity remains elusive. Our study indicates that UCP2, PGC-1α and p38 MAPK are all implicated in oxidative stress response caused by high glucose treatment in renal tubular epithelial cells.

CONCLUSION

We found that UCP2 plays a pivotal role in regulating PGC-1α, p38 MAPK and ROS. On one hand, UCP2 inhibits mitochondrial ROS production directly by uncoupling the oxidative phosphorylation from ATP production , On the other hand, UCP2 could further diminish the production of ROS by regulating the activity of PGC-1α and p38 MAPK (Figure 6). Hence, UCP2 represents an important target for new strategies to combat diabetic kidney disease. Future studies are needed to clarify the detailed mechanisms in these regulations.

CONFLICT OF INTEREST

The authors declare that there are no competing interests.

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