Rap1 ameliorates renal tubular injury in diabetic nephropathy

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Running title: Rap1b in diabetic nephropathy

Abbreviations: Diabetic nephropathy - DN, GTP-binding proteins - GTPase,
High glucose - HG, Extracellular matrix - ECM.

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Words number: 4598

Figures: 7

Supplements materials
Including 5 of Figures, 1 of Table

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ABSTRACT

Rap1b ameliorates high glucose (HG)-induced mitochondrial dysfunction in tubular cells. However, its role and precise mechanism in diabetic nephropathy (DN) in vivo remains unclear. We hypothesize that Rap1 plays a protective role in tubular damage of DN by modulating primarily the mitochondria-derived oxidative stress. The role and precise mechanisms of Rap1b on mitochondrial dysfunction and of tubular cells in DN was examined in rats with Streptozotocin (STZ)-induced diabetes that have Rap1b gene transfer using an ultrasound microbubble-mediated technique as well as in renal proximal epithelial tubular cell line (HK-2) exposed to HG ambiance. The results showed that Rap1b expression decreased significantly in tubules of renal biopsies from patients with DN. Over-expression of a constitutively active Rap1b G12V notably ameliorated renal tubular mitochondrial dysfunction, oxidative stress, apoptosis in the kidneys of STZ-induced rats, which was accompanied with increased expression of transcription factor C/EBP-β and PGC-1α. Furthermore, Rap1b G12V also decreased phosphorylation of Drp1, a key mitochondrial fission protein, while boosting the expression of genes related to mitochondrial biogenesis and antioxidants in HK-2 cells induced treated with HG. These effects were imitated by transfection with C/EBP-β or PGC-1α siRNA. In addition, Rap1b could modulate C/EBP-β binding to the endogenous PGC-1α promoter and the interaction between PGC-1α and Catalase or mitochondrial superoxide dismutase. Indicating that Rap1b ameliorates tubular injury and slows the progression of DN by modulation of mitochondrial dysfunction via C/EBP-β:PGC-1α signaling.
INTRODUCTION

Although glomerular injury is believed to initiate kidney damage in diabetic nephropathy (DN), recently emerging evidence suggests that tubular injury also plays a key role in the causation of damage in DN (1). Most of these studies have examined the tubular damage in the advanced stages of DN, but the mechanism(s) initiating tubular injury during this process have not been thoroughly explored.

Renal proximal tubule is uniquely susceptible to a variety of metabolic and hemodynamic factors, which is related to the events of apoptosis. Interestingly, increased apoptosis has been observed in the proximal and distal tubular epithelia in patients with diabetes (2), as well as in proximal tubular epithelial cells under high glucose (HG) ambience (3). Thus, it is believed that the events leading to apoptosis in tubular epithelial and further progression to tubulo-interstitial lesions are among the main features in DN (4). In addition, HG and angiotensin II could additively aid in the generation of reactive oxygen species (ROS), which may mediate renal tubular cell apoptosis (5). In addition, following the uptake of glucose metabolic intermediaries via various glucose transporters, the mitochondrial electron transport system is overwhelmed in proximal tubular cells, thus causing intracellular oxidative stress and cell damage(6). Indicating that HG itself is an initiating factor may be directly responsible for the causation of tubular damage and apoptosis in DN.
Nonetheless, the mechanism by which HG underpinning the mitochondrial dysfunction and tubular or tubulo-interstitial damage is unknown.

Rap1 (Ras-proximate-1 or Ras-related protein 1) is a small GTPase, which has been shown to regulate cell adhesion, migration, proliferation and cell survival. We previously demonstrated decreased activation of Rap1b under HG ambience \textit{in vitro} and found HG-induced mitochondrial dysfunction was rescued by over-expression of Rap1b in tubular cells (9). However, whether Rap1 can dampen the progression of DN \textit{in vivo} by modulating mitochondrial-derived oxidative stress is unclear, and it needs to be investigated along with the delineation of the signaling pathways that may be involved.

**RESEARCH DESIGE AND METHODS**

**Antibodies, plasmids and other reagents:** Polyclonal Anti-Rap1b antibody; polyclonal anti-phospho-Drp1 (Ser637) and (Ser656) antibodies, monoclonal anti-PGC-1α, anti-C/EBP-β from Cell Signaling; Human/Mouse/Rat cytochrome C monoclonal antibody from BD Biosciences, Pharmingen; pro-caspase3 antibody and pro-caspase 9 antibody from Thermo Fisher Scientific; monoclonal anti-cleaved Caspase-3 (Asp175), rabbit polyclonal IgG antibodies including anti-Mfn2, anti-Catalase, anti-Mn-SOD, anti-NRF1, anti-GSH-Px (Glutathione peroxidase) and anti-mtTFA (Santa Cruz...
Biotechnology); Plasmids containing pcDNA/Rap1b G12V and pcDNA/Rap1b S17N mutant were generated in our lab as previously described (10). ERK1/2 siRNA, PGC-1α siRNA, DFC and MitoRed and MitoSOX were purchased from Invitrogen.

**Morphological analysis of kidney:** Human kidney biopsy tissues were obtained from DN (DN =12) of 10-15 years duration, and equal number of non-diabetic patients (MN, N =12) were recruited for the study. The renal sections were stained with PAS and PASM. Tubulo- interstitial lesion index was determined using a semi-quantitative scoring system (11). Tubular damage was also scored (12) and the mitochondrial alterations in renal tubules were gauged by electron microscopy(EM) as previously described (13). The human experimental protocols as described above were approved by the Institutional Human Experimentation Ethics Committee, Second Xiangya Hospital, Central South University.

**Measurements of blood glucose. γ-glutamyl transpeptidase (γ-GT), β-N-acetyl-β-D-glucosaminidase (β-NAG) and Urine albumin excretion (UAE) levels:** Blood glucose was detected by a blood glucose monter (Boehringer Mannheim Inc, Germany). The γ-GT concentrations were measured using human gamma glutamyl transpeptidase, GGT ELISA kit (Biocompare) and Urine β−NAG was measured by automated colorimetric
method (Pacific Biomarkers, Inc). Urine albumin was measured by rat urine albumin ELISA kit (Bethyl Laboratories) and urine creatinine levels were tested using the QuantiChrom Creatinine Assay Kit (BioAssay Systems) following the manufacturer’s protocol. UAE was normalized with creatinine excretion and expressed per mg creatinine.

**Animal experimental design:** A total of 60 adult male Sprague-Dawley rats of 8 weeks age (BW = 210 - 230 g) were divided into 4 groups, 15 animals each. The 1\(^{st}\) group was injected with a normal saline only which served as a control. The 2\(^{nd}\) group of rats received a single dose of streptozotocin (STZ, 65 mg/kg, IP). The 3\(^{rd}\) group included rats with STZ-induced diabetes but injected with Rap1 V12G using ultrasound micro-bubble gene transfer technique (12). The 4\(^{th}\) group included rats with STZ-induced diabetes but injected with empty vector control (STZ + empty vector). All animals were sacrificed at 8 weeks following STZ administration. The animal experimental protocols as described above were approved by the Institutional Animal Experimentation Ethics Committee.

**Ultrasound-mediated gene transfer of inducible Rap1V12G gene-bearing micro-bubble into the rat kidneys:** To control Rap1V12G transgene expression within the kidney, a doxycycline-induced Rap1 V12G expressing plasmid was constructed as previously described (10, 14). Briefly, pTRE-Rap1b G12V was generated by subcloning a rat full length Rap1b G12V
cDNA into pTRE (Clontech, Palo Alto, CA), a tetracycline-inducible vector. An improved pTet-on vector (Clontech), pEFpurop-Tet-on was constructed as described previously (15,16). To achieve doxycycline-inducible Rap1 G12V transgene expression in the kidney, the ultrasound-microbubble-mediated system was applied as previously described (14,15). Briefly, 24 hrs following STZ injection, the left rat kidney was transfected with a mixture of pTR-Flag-Rap1b and pEFpurop-Tet-on with Optison (Mallinck rodt, St. Loui s, MO) in 1:1 vol/vol ratio. The mixture contained 25µ g of the each of the plasmids in 0.5 ml saline, and it was introduced via the left renal artery following a temporarily cessation of the renal blood supply for 3-5 min with a microclamp. Control animals received the vector without Rap1 G12V gene, and its transfection was monitored by immunohistochemistry(IHC), using a monoclonal antibody directed against Flag-M2. The Rap1b expression and activity within the kidney was assessed by quantitative real-time PCR, Western blot/Immunoprecipitation, as previously described (9, 10, 14).

**Measurements of superoxide generation and apoptosis:** Mitochondrial superoxide generation was detected by using a specific mitochondrial superoxide indicator, MitoSOX red (Molecular Probes, USA). Dihydroethidium (DHE) and 5, 6-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate (CM-DCF-DA, Wako) were used to assess the production of intracellular superoxide anion (O$_2^-$) and H$_2$O$_2$, respectively. TUNEL procedure was
employed to gauge apoptosis following vendors’ instructions.

**mtDNA Studies:** The mtDNA damage to high and low molecular weight DNA was evaluated as described previously (10,12). Briefly, PCR products were subjected to 1.6% agarose gel electrophoresis followed by staining with ethidium bromide to detect 8636- and 316-bp DNA products. For long PCR the primers were as follows: sense: 5’-AGTGCATACCGCCAAAAGA-3’ and antisense: 5’-TCTAGAGCCCACTGTAAAG-3’. The primers for short PCR were as follows 5’-ATGGTCTGAGCTATGATATCAA-3’ (sense) and 5’-GATTTTGGCTAGGTTGG-3’ (antisense).

**Cellular distribution of p-Drp1 and of mitochondrial cytochrome C:**
Confocal microscopy was performed to delineate the distribution/localization of p-Drp1 in ref(17). The expression of Drp-1 and cytochrome C in isolated mitochondria was assessed with Western blotting procedures.

**Assessment of mitochondrial transmembrane potential (Δψm):** HK-2 cells were transfected with Rap1b, C/EBP-β-siRNA or PGC-1α-siRNA. Then they were treated with HG, and then 10 nM of TMRE dye (Molecular Probes) added to the medium for 10 minimums. The mitochondrial Δψm in intact cells was assessed by FACS analyses and Confocal microscopy using a wavelength of 582 nm. In isolated mitochondria from renal tissues, the Δψm was gauged
following a load of rhodamine 123 (Rh123), Δψm was calculated as discussed previously (18,19).

**Mitochondrial enzyme activities:** Manganese superoxide dismutase (Mn-SOD) activity and CuZn-SOD activity was determined using Superoxide Dismutase [SOD] Activity Assay Kit (Alexis® Biochemicals). The catalase activity was measured using Catalase Activity Colorimetric/Fluorometric Assay Kit (BioVision, Inc), and glutathione peroxidase (GSH-Px) were determined using the Glutathione Peroxidase (GSH-PX) Assay Kits (Biocompare, Inc) following the vendors' guidelines.

**Examination of fragmentation and length of long-axis of mitochondria:**
EM processing used to determine mitochondrial fragmentation and several contiguous (side-by-side) digital images were generated. Percentage of cells that had less than 1% long filamentous mitochondria were reflective of mitochondrial fragmentation. To determine the length of long-axis of mitochondria, digital images were generated. Then, the length of individual mitochondria in a cell was measured as ref (20,21).

**Assessment of mitochondrial H₂O₂ production and of mitochondrial permeability transition pore (MPTP) following Ca⁺⁺ load:** Mitochondrial H₂O₂ production rate was evaluated using Scopoletin fluorescence, as
described previously (22). In addition, the MPTP was evaluated by Ca\textsuperscript{2+} load method using Mitochondria Calcium Fluorescence Detection Kit by following the manufacturer's guidelines (Genmed Scientifics).

**PGC-1α gene promoter analysis:** Various deletion constructs of PGC-1α promoter were generated by PCR. The PCR products were cloned into XhoI and Hind III-digested pSEAP2-Enhancer plasmid vector (CLONTECH). Minimal promoter activity of the PGC-1α promoter activity was measured in the supernatants of the HK-2 using a Great EscAPE\textsuperscript{TM} SEAP fluorescence detection kit (CLONTECH). The highest promoter activity was designated as being 100% like in ref (23,24).

**Nuclear extract preparation and electrophoretic mobility assays (EMSA):**
Nuclear extracts used in EMSAs assays from HK-2 cell were performed, as described in previous publications (25). Briefly, the nuclear extracts (10µg) were incubated with 40,000 CPM of [γ\textsuperscript{32P}] dATP end-labeled oligonucleotides containing putative CRE (cAMP response elements) within the -146 to -132 bp region of the hPGC-1α promoter corresponding to GGCTGCCTTTGAGTGACGTCA CAC-3'. The samples were then subjected to native 5% acrylamide gel electrophoresis and imaged.

**ChIP analysis:** ChIP analysis was performed using a transcription factor ChIP
kit following the instruction manual (Diagenode). The primer sequences spanning -2160 to -1938 region were as follows: Sense - 5’-GGCTTCTGGTTCGCCTGCTC AGU3’ and antisense-5’-ATACTGATAGCTGCGATTGTTAAGCGU3’. This region of amplification contains FoxO1-dependent binding element (FoxO1-DBE) in the Catalase enzyme promoter. The PCR was also performed for MnSOD with following sequences as: Sense-5’-GTTCTCTCTCGCTGAC TGTT-3’ and antisense 5’-CTGAA CCGTTTCC GTTGC TT-3’.

**RESULTS**

**Decreased expression of Rap1b in renal tissues of patients with DN:**

Morphological changes in both the glomerular and tubulo-interstitial compartments, including focal tubular atrophy and interstitial fibrosis, were highlighted by PASM and PAS staining in DN patients compared to non-Diabetics patients (N-DN). IHC staining revealed a significantly decreased Rap1b expression in the renal tubules of DN patients compared to that of N-DN (Fig. 1A). Quantitatively, Rap1b staining intensity was decreased by >50% in renal tubules of DN patients (Fig. 1B). By EM, notable deformation of renal tubular mitochondria was observed in DN patients (Fig. 1Ah). Also, increased blood glucose and Scr levels were observed in DN (Figs. 1C and D). In addition, length of long-axis of mitochondria in tubular cells of renal biopsies from patients with DN was measured and it showed its shortening in DN compared to N-DN (Fig. 1E). Further analysis revealed an inverse correlation
between Rap1 expression and the tubulo-interstitial damage and urinary β-NAG levels (Figs. 1F and 1G).

**Rap1b-mediated protective effect on tubular injury in STZ-induced diabetic rats:** By IHC, Rap1b expression was predominantly localized to renal proximal tubules and it was notably decreased in renal tubules of rats with STZ-induced diabetes. While Rap1b expression markedly increased after ultrasound-mediated gene transfer, and this was even much higher than constitutively expressed in control rats (Fig. 2A). Western blot and Real-time PCR analysis revealed decreased mRNA and Protein expression of Rap1b in STZ rats, while its expression was apparently high in Rap1b G12V treated group (Figs. 2B, 2C and 2D). Although hyperglycemia was not changed in STZ-induced diabetic rats that received Rap1bG12V (Fig. 2E), The rats UAE levels were dramatically decreased following Rap1bG12V gene transfer in diabetic rats (Fig. 2F). The tubular damage was reflected by a significant increase in the urinary excretion of γ-glutamyltranspeptidase (γ-GT) and β-NAG in rats with STZ-induced diabetes, while they were substantially reduced by intra-renal injection of Rap1b G12V (Figs. 2G & 2H).

**Rap1b inhibit renal tubulo-interstitial fibrosis and oxidative stress, apoptosis in kidneys of rats with STZ-induced diabetes:** Compared to the control, an increase in the mesangial matrix and an expansion of the
tubulo-interstitial compartment was observed in kidneys of rats with STZ-induced diabetes over a period of 8 weeks. These morphological alterations were ameliorated by the administration of Rap1bG12V in STZ diabetic rats. In addition, there was a notable increase in Collagen I (Col-I) and fibronectin (FN) expression in kidneys of diabetic rats, while it was markedly reduced following Rap1bG12V injection (Figs. 3A-3C). These results were confirmed by Western blot analysis (Figs. 3D-3F). On the other hand, ROS production increased in renal proximal tubules of STZ rats as assessed by ROS-sensitive vital dye DHE. With over-expression of Rap1b, the ROS generation was significantly reduced. Like ROS generation, over-expression of Rap1b dramatically reduced the degree of apoptosis in cortical tubules of STZ rats by TUNEL assay with both fluorescence (TUNEL-F) and histochemical (TUNEL-H) procedures (Figs. 3G-3J).

**Over-expression of Rap1b inhibits peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and CCAAT/enhancer binding protein (C/EBP-β) expression, regulation of mitochondrial morphologic and functional changes in tubular cells of diabetic rats:** By SSH-PCR, PGC-1α mRNA level was notably decreased in renal tubule in STZ induced rat kidneys compared to control (figure not included). Furthermore, by immuno-staining showed that PGC1α and C/EBP-β were mainly expressed in renal proximal tubules, and their expression notably reduced in STZ rats.
kidneys compared to the control. However, following the administration of Rap1bG12V, their expression was normalized (Fig. 4A). Western blot analysis confirmed the above IHC findings (Figs. 4B, 4C and 4D). In addition, marked changes in mitochondrial morphology were seen in renal tubules of STZ-induced diabetic rats. The mitochondria were often angulated and attenuated along their longitudinal axis, some were swollen and had dilated cristae in DN rats. Moreover, frequent “cristolysis” with focal disruption of the inner mitochondrial membranes was observed. While with Rap1b administration, the mitochondrial morphology was partially restored, and angulations and attenuation was less frequently seen (Fig. 4E). The length and mean area of mitochondria were also reflected by the 2D EM analysis in mitochondrial fragmentation (Fig.4F) along with morphometric analysis of their area (Fig. 4G). Since ROS formation related to the functionality of MPTP, we measured Ca$^{2+}$ load to assess the MPTP in mitochondria isolated from renal tubule. The exposure of mitochondria to Ca$^{2+}$ (500 µmol/l) induced a relatively increased size of mPTP opening in STZ group compared to that of control. The opening was reduced to a certain extent with Rap1bG12V administration (Fig. 4H). In addition, a decreased mitochondrial membrane potential (MMP) and increased H$_2$O$_2$ was observed in mitochondria of rat renal proximal tubules of STZ-induced diabetes and it was reversed by Rap1b administration (Figs. 4I and 4J).

The expressions of pro-caspase 3 and pro-caspase 9 in the cytoplasm of
tubular cells of diabetic kidneys were decreased as compared to control, but they were restored following Rap1bG12V administration. In contrast, the expression of cleaved-caspase 3 was significantly increased and it was partially blocked with the treatment of Rap1bG12V. Furthermore, the expression of mCyto C and p-Drp-1 increased in cytoplasm of tubular cells of diabetic kidneys, which were normalized following Rap1bG12V administration. Furthermore, the expression of mCyto C was decreased and p-Drp-1 expression increased in mitochondrial fraction from tubules in rats with STZ-induced diabetes, both of them were normalized with Rap1bG12V administration (Figs. S1A, S1B and S1C). Real-time PCR (Fig. S1D) and Western blot analysis (Figs. S1E & S1F) also revealed a notable decrease in mRNA and mitochondrial protein expression of Catalase, Mn-SOD and GSH-Px in kidneys of diabetic rats compared to control. Similarly, the expression of nuclear respiratory factor-1 (NRF-1) and of mitochondrial transcription factor A (mtTFA), which are relevant to mitochondrial biogenesis, were also decreased in kidneys of diabetic rats. Their expressions were normalized by the over-expression of Rap1G12V. The studies were further extended to assess the relative enzyme activities of Catalase, Mn-SOD, CuZn-SOD and GSH-Px. They were also reduced in diabetic conditions, while they were normalized with the administration of Rap1G12V (Fig. S1G).

**High glucose inhibits Rap1 activity in HK-2 cells, and overexpression of Rap1 modulates PGC-1α expression as well as regulates mitochondrial**
dysfunction via ERK1/2-C/EBP-β pathway:  
Rap1b GTP activity decreased significantly in HK-2 cells exposed to HG (Fig. S2). In addition, PGC-1α mRNA expression was notably increased in HK-2 cells transfected with Rap1bG12V by SSH-PCR (Fig. 5A), which was confirmed by Northern blot analysis, while over-expression of Rap1b was found to rescue HG-reduced mRNA expression of PGC-1α in HK-2 cell (Fig.5B). Next, we investigated whether Rap1b-ERK1/2-C/EBP-β signaling pathway is involved in PGC-1α modulation. By Real-time PCR analysis, a dose-dependent decrease in the PGC-1α mRNA expression in HK-2 cells was observed under HG, and it was normalized by transfection of Rap1b G12V (Fig. 5C). The effect was partially inhibited by concomitant transfection with ERK1/2 or C/EBP-β siRNA. This effect was additive with the transfection of both siRNAs at the same time (Fig. 5C). Similar to mRNA, decreased in PGC-1α protein expression was observed under HG. The effect was negated by Rap1bG12V transfection. The inhibitory effect by ERK1/2 or C/EBP-β siRNA was also reflected in HK-2 cells, as assessed by the immunofluorescence microscopy (Figs. 5D and 5E) and Western blot analyses (Fig. 5F). Furthermore, a notable decrease of C/EBP-β nuclear translocation was observed in HK-2 cells exposed to HG. With the transfection of Rap1b, the nuclear translocation of C/EBP-β was restored, while it was inhibited by ERK1/2 siRNA (Figs. 5G and 5H).

We also assessed whether Rap1b modulates mitochondria membrane potential (ΔΨm) and mtDNA fragmentation via C/EBP-β:PGC-1α pathway in
HK-2 cells subjected to HG. A loss of TMRE associated fluorescence (indicative of $\Delta \Psi_m$) was observed in HK-2 cells subjected to HG in a time- and concentration-dependent manner. This loss was restored in cells transfected with Rap1bG12V. However, this restorative effect was partially blocked following the treatment of either C/EBP-β siRNA or PGC-1α siRNA (Figs. S3A, 3B & 3C). In addition, Real-Time PCR showed that exposure of HK2 cells to HG induced a dose-dependent and time-dependent increase in mtDNA fragmentation. Transfection of Rap1b G12V partially inhibited the mtDNA damage. While C/EBP-β or/and PGC-1α siRNA blocked the protective effect of Rap1bG12V on the mtDNA damage (Figs. S3D & S3E).

Rap1b inhibits mitochondrial ROS production, cytochrome C release and apoptosis, regulate the expression and activity of anti-oxidative genes, and phosphorylation of drp1 in HK-2 cells via C/EBP-β:PGC-1α pathway: Confocal images delineated that HG increased both mitochondrial ROS and total ROS production, while it was inhibited by over-expression of Rap1bG12V, and C/EBP-β or/and PGC-1α siRNA partially blocked the effect of Rap1b on the ROS production induced by HG in HK2 cells (Figs. 6A & 6B). The FACS analysis revealed that HG caused a decreased cell survival associated with increased apoptosis, which could be reversed by over-expression of Rap1bG12V, while inhibition of C/EBP-β or/and PGC-1α by siRNA negated Rap1b protective effect (Fig. 6C). In addition, HG decreased the expression of
mCyto C in mitochondrial fraction and increased its expression in cytosolic fraction. With Rap1b transfection, its release was significantly inhibited. Accompanied with mCyto C release, pro-caspase-3 and pro-caspase-9 protein expression decreased in the cytoplasmic compartment of HK-2 cell with HG treatment. In contrast, the expression of cleaved caspase-3 was increased in the cytoplasm of HK-2 cell exposed to HG ambience. These alterations were normalized in HK-2 cells transfected with Rap1bG12V, but partially blocked with C/EBP-β or/and PGC-1α siRNA transfection (Fig. 6D & 6E). In addition, over-expression of Rap1G12V in HK2 cells ameliorated HG-induced reduction in mRNA levels of Catalase, Mn-SOD, NRF-1 and mtTFA; and these effects were blocked by the pre-treatment of PGC-1α and C/EBP-β siRNA. Similar changes were seen for their protein expression and ROS scavenging enzyme activity (Fig. S4).

In addition, HG ambience increased the phospho-Drp1 (p-Drp1 at Ser637) in HK-2 cells; while this effect was abolished by Rap1bG12V transfection (Fig. 6F). However, transfection with Rap1b mutant construct (Rap1b S17N) failed to inhibit p-Drp1 expression in HK-2 cells under HG. Associated with the change in p-Drp1 expression, the mitochondria became relative more fragmented in a time-dependent manner under HG, and this process could be inhibited by over-expression of Rap1bG12V (Fig. 6G). Moreover, increasing frequency of cells undergoing apoptosis in a time-dependent manner induced by HG was found, as assessed by cytometric
analysis. The extent of cells undergoing apoptosis was reduced by over-expression of Rap1bG12V (Fig. 6H). Western blot analyses indicated an imbalance induced by HG between fusion and fission events of mitochondria, as reflected by increasing the protein expression of p-Drp-1 and decreasing expression of Mfn2; and these changes were reversed with the over-expression of Rap1b but not with Rap1bS17N mutant transfection. Interestingly, ERK1/2 siRNA could also partially block the normalizing effect of Rap1bG12V (Fig. 6I).

**Modulation of PGC-1α promoter activity by Rap1b:** A ~2.0 kb fragment upstream of open reading frame (ORF) of human PGC-1α was isolated and cloned into pSEAP-1. Five deletion constructs spanning different regions of PGC-1α promoter were generated and subcloned into pSEAP1-enhancer vector. The highest SEAP activity was observed in the deletion construct spanning +28 to -1136 bp upstream of the ORF (Fig. 7A). This construct was used for the subsequent experiments. HG ambience inhibited the activity of PGC-1α promoter in a dose-dependent manner (Fig. 7B). Transfection of Rap1bG12V restored activity of PGC-1α promoter under HG, almost close to basal conditions. This restorative effect was partially abolished with the concomitant transfection of ERK1/2 or C/EBP-β siRNA (Fig. 7C), suggesting that the protective effect of Rap1b on the HG-inhibition of PGC-1α promoter activity is dependent upon the ERK1/2:C/EBP-β pathway.
To verify whether Rap1 regulation of PGC-1α promoter activity is related to binding of transcription factor C/EBP-β to PGC-1α DNA, EMSA was carried out. The binding of the C/EBP-β oligo was noted to be reduced in the nuclear extracts from HK-2 cells subjected to HG. However, transfection of Rap1bG12V could partially normalize the band density, while it was reduced in cells treated with C/EBP-β siRNA. The super shift observed by the use of anti-PGC-1α antibody confirmed the specificity of EMSA experiments (Fig. 7D).

We also employed ChIP assay coupled with PCR to determine the interaction of PGC-1α with the Catalase or Mn-SOD promoter region in HK-2 cells. These results support the notion that the PGC-1α could physically interact with the potential binding sites of FoxO1-DBE in both Catalase or Mn-SOD promoters. The band densities of PCR products remarkably decreased following treatment with HG. Mannitol had no effect. The over-expression of Rap1bG12V restored the band density to basal levels (Fig. 7E), while they remained reduced as seen in that of HG with the transfection of Rap1bS17N mutant or co-transfected with Rap1bG12V and C/EBP-β siRNA (Fig. 7F).

**DISCUSSION**

It is known that substitution of Glycine at residue 12 to Valine (GGC → GTC) alters the GTPase activity (26). This results in constitutive activation of Rap1b to enhance the mitogenic response mediated by cAMP while maintaining
cellular differentiation (27). Thus, the Rap1bG12V mutant construct was used in this study to delineate its effect in regulation of tubular injury in diabetic state. To modulate and over express Rap1G12V transgene inducible expression within the kidney, a doxycycline-induced Rap1G12V expressing plasmid was constructed by using Tet-On system. Ultrasound microbubble-mediated transgene (UMMT) method was used for the delivery of these plasmids. UMMT is a novel, nonviral, effective and safe method for delivering drugs or genes to target organs or cells (12). This technique has been used to observe the role of Smad7 in STZ induced DN for 5 weeks (28). In this study, we used this procedure to transfer Rap1b G12V gene into the rat kidney, then kidneys were harvested and utilized for IHC, Northern and Western blot analysis. We found that over-expression of Rap1bG12V could reverse the changes in renal tissues induced by hyperglycemia and rectify the UAE, γ-GT and β-NAG, and decreased the expression of Col1 and FN in the kidneys of STZ-induced Rats (Figures 3 & 4), suggesting that Rap1bG12V exerts a beneficial effect in tubular injury in DN. The pathogenesis of DN is multifactorial, the dominant being ROS-mediated injury.

Mitochondrial dysfunction could be a contributing factor to the pathogenesis and complication of diabetes mellitus (29). Mitochondrial-mediated pathway leading to apoptosis is one of the most important cell death signaling which causes release of mCyto C, activation of caspases-9 and -3 leading ultimately to apoptosis (30). Interestingly, transient
transfection of constitutively active Rap1G12V into C2C12 myotubes lead to a partial rescue of simvastatin-induced inhibition of mitochondrial respiration (31). Here, we observed that over-expression of Rap1bG12V could reverse ROS generation and apoptosis by protecting mitochondrial dysfunction in the renal proximal tubules of diabetic rats and in cultured tubular cell induced by HG.

PGC-1α may exert a rescuing effect in preserving the mitochondrial function and maintain homeostasis of oxidative metabolism (32). Its expression seems to be regulated by C/EBP-β, a cAMP regulated transcription factor (33). A decreased PGC-1α expression in muscle tissues of patients with diabetics may be responsible for decreased expression of NRF-dependent metabolic and mitochondrial genes (34). Furthermore, PKA:C/EBP-β pathway plays a critical role in the regulation of PGC-1α expression (35) and ERK1/2 activation is essential for cAMP-dependent C/EBP-β activation, which can phosphorylate and activate C/EBP-β (36). In this study, we demonstrate that over-expression or over-activity of Rap1b could up-regulate HG-induced reduction of PGC-1α mRNA and protein expression, while reduce its nuclear translocation by ERK1/2 or C/EBP-β siRNA pathway in the kidneys of STZ rats and HK-2 cells induced by HG.

PGC-1α is also a master regulator of ROS scavenging enzymes including Mn-SOD2, Catalase, GSH-Px and the uncoupling protein 2 (37). Reduction of PGC-1α expression may promote oxidative stress (38). In this study, activation of Rap1 regulates mitochondrial ROS production by
increasing anti-oxidative enzymes gene expression modulating via ERK1/2, C/EBP-β and PGC-1α signaling pathway in the kidneys of diabetic animals. In addition, PGC-1α has a major impact in regulation of mitochondrial DNA replication and inducing gene expression for NRF-1, NRF-2 and mtTFA by interacting with NRF-1 and thereby co-activating its transcriptional activity (39). NRF1 may regulate nuclear genes encoding respiratory subunits and components of mitochondrial transcriptional and replication machinery (40). We also noted that over-expression of Rap1b could also regulate the gene and protein expression of mtTFA and NFR1 in diabetic kidney tissues via C/EBP-β: PGC-1α signaling.

A perturbation in the balance between mitochondrial fission proteins Drp1/DLP1/Dnm1 and mitochondrial inner membrane fusion protein mitofusin2 (Mfn2) leads to mitochondrial fragmentation (41). Under HG ambience the mitochondria are relatively small, compact with decreased expression of Mfn1 while increase in Drp1 (42), which is likely to lead to mitochondrial and cellular dysfunction (43). In addition, ERK is believed to be one of the intracellular regulator of signaling for the Drp-1 (44) and Rap1b is known to play a role in cell adhesion, migration and tubule formation by coupling to ERK (45). Here we observed that Rap1 regulates mitochondrial dysfunction induced by HG via the inhibition of phosphorylation of Drp1 dependent of ERK 1/2 signaling pathway.

It is known that C/EBP-β binds to CRE in the PGC-1 α promoter at -756
to -752 bp, which is essential for PGC-1α activation (46). Here we also demonstrated that Rap1bG12V could reverse HG reduced binding with C/EBP-β at the CRE site of PGC-1α promoter. On the other hand, Foxo1 has been described to protect pancreatic β-cells against oxidative stress induced dysfunction (47). PGC-1α can interact with the regulatory promoter sequences of Mn-SOD and Catalase through FoxO1 (48). To further investigate whether Rap1 regulates expression of oxidative stress protective genes, ChIP experiments were performed. PGC-1α binding to Catalase and Mn-SOD promoter at FoxO1-DBE sites was reduced under HG ambience in HK-2 cells, while over-expression of Rap1 could reverse this effect via C/EBP signaling.

In conclusion, it seems that hyperglycemia inhibits Rap1 expression and its activity. This leads to tubular cells injury in patients with DN and STZ-induced diabetic animal models. This mechanism involves a decreased PGC-1α expression via ERK1/2: C/EBP-β pathway and phosphorylation of Drp-1, thus inducing an imbalance between mitochondrial fission and fusion proteins, which is followed by a series of events leading to overproduction of ROS and tubular apoptosis (Fig. S5). These events are reversed with the over-expression of Rap1b, suggesting activation of Rap1b could decelerate the progression of DN by regulation of mitochondrial dysfunction via ERK-C/EBP-β: PGC-1α signaling pathway.
AUTHOR CONTRIBUTIONS: L X, X Z, S Y, D Z, J L and P S generated the data for the manuscript. F L, Z Z, M Z and P X discussed the results of the manuscript. L X, X Z partially wrote the manuscript. Y S K and L S edited the manuscript.

ACKNOWLEDGMENTS This work was Supported by grants from the Creative Research Group Fund of the National Foundation Committee of Natural Sciences of China (30971379, 81270812, 81370832), Doctoral Fund of Ministry of Education of China (20110162110012), Furong Scholars Fund from Hunan Province Education Department, National Basic Research Program of China 973 program (No.2012CB517601), Program for Changjiang Scholars and Innovative Research Team in University (IRT1195) and a Grant from the NIH, DK60635. Dr. Lin Sun is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

CONFLICT OF INTEREST STATEMENT: NO conflict of interest to disclose.
REFERENCES


FIGURE LEGENDS

Figure 1. Decreased expression of Rap1b in renal tissues of patients with diabetic nephropathy.

(Panel A) PASM and PAS staining show tubular atrophy and interstitial fibrosis in renal biopsies in patients with DN (panel a, b, c and d) (Magnification X100). Immunohistochemical studies revealed decreased expression of Rap1b in DN patients (panel f versus e). By electron microscopy, notable deformations were seen in the tubular mitochondria of renal tissue of DN patients compared to N-DN (panel h versus g) (magnification X10,000). (Panel B) Averaged relative intensity following staining with of anti-Rap1b antibody of kidney biopsies of DN versus N-DN patients (*P <0.01). (Panels C & D) Serum creatinine (Scr) and blood sugar levels in DN and N-DN patients (*P<0.01).

(Panel E) Relative percentage of renal tubular cells with elongated mitochondria in diabetic versus non-diabetic patients. (Panels E & F) Scatter plots show relationship between Rap1b expression and tubular interstitial damage and urinary N-acetyl-β-D-glucosaminidase (β-NAG) levels. Values are means +/- SE, * <0.01 versus N-DN groups.

Figure 2. Expression of exogenous Rap1b in kidney tissues and its effect on blood glucose, urine albumin excretion (UAE) and urinary
excretion of γ-glutamyl-transpeptidase (γ-GT) and β-N-acetyl-glucosaminidase (β-NAG) in the STZ induced Rats.

(Panel A) By immuno-histochemistry Rap1b expression was assessed in control (A-a), STZ Rats (A-b), STZ+Rap1b V12G Rats (A-c) and STZ + empty vector only group (A-d) of rats (Magnification X400). By Real time PCR (Panel B) and Immunoprecipitation/ Western blot analyses (Panel C), a decreased mRNA expression and Rap1b GTP-activity was seen in kidneys of STZ Rats, which was reversed by injection of Rap1bG12V. (Panel D) The bar graphs represent the expression of the Rap1b GTP relative to β-actin. (Panel E) Blood glucose concentration in each group. (Panel F) Rats UAE levels. (Panels G & H) Urinary excretion of γ-GT and Urine β-NAG levels. Values are means +/- SE, * P <0.01 versus to control, # P <0.01 versus to STZ.

Figure 3. Effect of Rap1b G12V on renal morphology, ECM expression, oxidative stress and apoptosis in rats kidneys with STZ-induced diabetes.

(Panel A) Kidney sections were stained with PAS (A, upper panels) and with anti-Collagen-1 (Col-1, middle panels) and-fibronectin (FN) antibodies (lower panels) (magnification X 400). (Panels B & C) represent semi-quantification of IHC staining of Col-1 and FN. (Panel D) By Western blot analysis, Rap1bG12V inhibited the expression of Col-1 and FN in rats with STZ-induced diabetes.
(Panels E & F) Quantification of average band density calculated from different Western blots. (Panel G) Increased oxidative stress and apoptosis was seen in tubular cells of diabetic rat kidneys, as assessed by DHE and TUNEL-fluorescence (TUNEL-F) or -histochemical (TUNEL-H) staining, while the effect was reduced by transfection of Rap1bG12V plasmid into the kidney. (Panels H & J) Quantification of tissues stained with DHE, TUNEL-F and TUNEL-H procedures. Values are means +/- SE, *P <0.01 versus control; #P <0.01 versus STZ, N =6.

**Figure 4** Effect of overexpression Rap1b on PGC-1α and C/EBP-β protein expression and the hyperglycemia-induced altered mitochondrial morphology and function in diabetic rat kidneys.

(Panel A) Immuno-histochemical studies revealed a decreased in situ expression of both PGC-1α and C/EBP-β in the respective cytoplasmic and nuclear compartments of renal tubules in kidneys of rats with STZ-induced diabetes. Similar expression patterns were seen by Western blotting analyses (Panel B). (Panels C & D) Quantification of average band intensity of Western blots. (Panel E) By electron microscopy deformation of mitochondria with dilatation of the cristae was seen in kidneys of rats with STZ-induced diabetes. With Rap1b overexpression aberrant cristae was reduced, but residual swelling of mitochondria was seen (asterisks). (Panel F) Relative percentage
of tubules with elongated mitochondria in diabetic versus non-diabetic rats and following Rap1b transfection. (Panel G) Bar graphs depict mitochondrial area per square micrometer in tubular cells of the kidney in four groups. (Panel H) Ca++-induced mitochondrial permeability transition pore (mMTP) opening, Mitochondrial membrane potential (Panel I), and H$_2$O$_2$ production (Panel I). Values are means +/- SE, *p <0.01 versus control, #p <0.01 versus STZ groups.

**Figure 5. Effect of Rap1b on PGC-1α and C/EBP-β nuclear translocation in HK2 cells under high glucose ambience.**

(Panel A) SSH-PCR shows that up-regulated expression of PGC-1α mRNA in HK-2 cell transfected with Rap1G12V. Northern Blot analyses (Panel B) and Real time PCR analyses (Panel C) showed overexpression of Rap1 G12V reversed the HG-induced reduction in PGC-1α mRNA expression; while pre-treatment of HK-2 cells with ERK 1/2 siRNA or C/EBP-β siRNA negated this reversal effect. (Panel D) Photomicrographs showing that HG decreases nuclear translocation of PGC-1α, while over expression of Rap1bG12V normalized the nuclear translocation. (Panel E) Prior treatment of HK-2 cells with C/EBP-β or ERK 1/2 siRNA partially negated the Rap1bG12V-related restorative effect. (Panel F, F-1) Western blot analysis showed PGC-1α expression of nuclear protein in HK-2 cells. (Panel F, F-2) Quantification of
average band intensity from four separate Western blots. (Panel G) Cellular immunofluorescence showing that overexpression of Rap1bG12V blocks the HG induced inhibition of nuclear translocation of C/EBP-β in HK-2 cells, while the effect was abolished in cells transfected with ERK1/2 siRNA. These results were confirmed by Western blot analysis (Panels H, H-1 & H-2). Bar graph represents the quantification of average band intensity. Values are means +/− SE, *p <0.01 versus 5 mM D-glu, ²p <0.01 versus 30 mM D-glu, ³p <0.01 versus 30 mM D-glu + Rap1b.

Figure 6 Overexpression of Rap1b inhibits generation of ROS, mCyto C release from mitochondria, decreases apoptotic protein expression and mitochondrial altered dynamics in HK-2 cells induced by high glucose.

(Panel A) Confocal images reveal the levels of mitochondrial ROS and intracellular ROS in HK-2 cells. (Panel B) The bar graphs represent a summary of FACS experiments of mitoSOX (Panel B-1) or DFC (Panel B-2) studies. (Panel C) FACS analyses depict cell survival. (Panel D) Western blots of Cytosolic proteins show an altered expression of mCyto C, cleaved caspases-3 and pro-caspase 3 and 9. (Panel E) The bar graphs represent the quantification of average band intensity of panel D. (Panel F) Confocal microscopy of HK2 cells stained with mitotracker (red) and anti-pDrp (Ser637) antibody (green). (Panel G) Mitochondrial fragmentation in HK2 cells treated 5
and 30 mM D-glucose for 24~168 hours. (Panel H) Flow cytometric analyses with FITC Annexin V staining. (Panel I) Western blot analyses of mitochondrial extract from HK-2 cells and Bar graphs represent the band intensity. Values are means +/- SE, *p < 0.01 versus 5 mM D-glu, &p < 0.01 versus 30 mM D-glu, #p < 0.01 versus 30 mM D-glu +Rap1b.

**Figure 7. Effect of Rap1b on PGC-1α promoter activity, C/EBP-β binding to PGC-1α promoter and interaction between PGC-1α promoter with promoters of Catalase and Mn-SOD**

(Panel A) Five deletion constructs spanning different regions of PGC-1α promoter subcloned into pSEAP1-enhancer vector and their respective activities. (Panel B) Promoter analysis in DC4 construct following various treatments. (Panel C) Transfection of Rap1bG12V plasmid individually led to a 40% - 50% increase in the SEAP activity under HG ambience, while blocked partially with the co-transfection of ERK1/2 siRNA or PGC-1α siRNA. (Panel D) EMSA assay show C/EBP-β binding in human PGC-1α promoter in samples isolated from nuclear extracts of HK-2 cells subjected to HG. While a significant reversal in the band intensity was seen following transfection with Rap1bG12V construct. The reversal effect was not seen following transfection of Rap1bS17G mutant or C/EBP-β siRNA. (Panel E) ChIP assay yielded a 254-bp (Catalase) and 237-bp (Mn-SOD) products with decreased band
intensity in anti-PGC-1α antibody immunoprecipitated nuclear material from
cells subjected HG compared to that of 5 mM glucose. The decreased intensity
was restored in cells transfected with Rap1bG12V, while this effect was
abolished with the transfection of Rap1bS17G mutant or C/EBP-β siRNA
(Panel F).
Figure 1. Decreased expression of Rap1b in renal tissues of patients with diabetic nephropathy.
Figure 2. Expression of exogenous Rap1b in kidney tissues and its effect on blood glucose, urine albumin excretion (UAE) and urinary excretion of γ-glutamyl-transpeptidase (γ-GT) and β-N-acetyl-glucosaminidase (NAG) in the STZ induced Rats.
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270x405mm (300 x 300 DPI)
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225x350mm (600 x 600 DPI)
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Figure 7. Effect of Rap1b on PGC-1α promoter activity, C/EBP-β binding to PGC-1α promoter and interaction between PGC-1α promoter with promoters of Catalase and Mn-SOD
Online Appendix

1. SUPPLEMENTAL FIGURES
Figure S1. Rap1 inhibits Cyto C release, Drp1 translocation into the mitochondria, modulation of pro-caspases 3 and 9, and cleaved-caspase 3 expression and anti-oxidative genes in kidneys of STZ induced diabetes.

(Panel A) Western blots of tubular extracts of kidney tissues in each group showing protein expression of pro-caspase 3, cleaved-caspase 3 and pro-caspase 9, mCyto C and phosphorylation of Drp-1 (p-Drp-1 at Ser656). (Panel B) Western blots of mitochondrial fractions from kidney tissues showing protein expression of mCyto C and p-Drp1 at Ser656. (Panel C) The bar graphs represent the quantification of average band intensity of panel A (A1 – A5) and Panel B (B1 & B2), N=4. (Panel D) By real time PCR showing mRNA expression of Catalase, MnSOD, GSH-Px, RNF1 and mtTFA in kidneys of each group. (Panel E) Western blots of mitochondrial fractions from kidney tissues showing protein expression of Mn-SOD, Catalase, GSH-Px, NRF1 and mtTFA. (Panel F) Quantification of average band density from four Western blot analyses included in Panel E. (Panel G) Relative activities of antioxidant enzymes (Catalase, Mn-SOD, CuZn-SOD and GSH-Px) in kidney tissues in various groups. *P <0.01 versus control, # <0.01 versus STZ group.
Figure S2. Decreased expression of Rap1b and Rap1b GTP activity in HK-2 cells exposed to high glucose ambience.

(Panel A) By cell immunofluorescence microscopy a dose-dependent decreased expression of Rap1b in HK-2 cells treated with high glucose was observed. Mannitol (30 mM) was used as an osmolality control. (Panels B & C) Western Blot/activity assay show that the expression of Rap1b GTP activity decreased in a dose and time-dependent manner in HK-2 cells treated with D-glucose. (Panels D & E) Bar graphs represent the quantification of relative band intensity of blots depicted in panels B & C. Values are means +/- SE, N =4, *P <0.05, **P <0.01 vs. control.
Figure S3. Effect of Rap1b on HG induced membrane potential (ΔΨm) and mtDNA fragmentation in HK2 cells.

(Panel A) Confocal microscopy of HK2 cells transfected with Rap1b G12V, CEBP-β siRNA and PGC-1-α siRNA, then subjected to different concentrations of D-Glucose for 168 hrs and then stained with TMRE. Lower panels depict the area outlined by rectangles of the upper panels. (Panel B) Flow cytometric analyses of HK-2 cells treated with different concentrations of D-glucose and then stained with TMRE. (Panel C) Overexpression of Rap1b G12V led to an amelioration of TMRE fluorescence to baseline, while pretreatment with C/EBP-β or PGC-1-α siRNA led to only a partial reversal of amelioration. (Panel D) High molecular weight mtDNA expression was measured by Real time PCR in cells subjected to different concentrations of glucose for 24, 96 and 168 hrs. (Panel E) Treatment of HG for 168 hrs induced increased DNA fragmentation, which was reduced with the transfection with Rap1b G12V; however, this normalizing affect was partially reduced with the pre-treatment of CEBP-β siRNA and PGC-1-α siRNA. *P <0.01 versus 5 mM-D-glucose; †P <0.01 versus 15mM D-glucose; ‡P <0.01, ‰P <0.05 versus 30 mM D-glucose + Rap1bG12V, N =6.
Figure S4. Effect of Rap1b on the expression of anti-oxidative genes, NRF-1 and mtTFA and their activity in HK-2 cells exposed to HG.

(Panel A) Real-time PCR analysis showing that overexpression of Rap1 G12V in HK2 cells ameliorates HG-mediated reduction in mRNA levels of Catalase, Mn-SOD, NRF-1 and mtTFA; while these effects were blocked with the pre-treatment of PGC-1α and C/EBP-β siRNA. (Panel B) Similar results were seen for protein expression, as assessed by Western Blotting procedures. (Panel C) The bar graphs representing the relative intensity of bands of Western blot analysis, N=4. (Panel D) The bar graphs representing enzyme activity of antioxidant proteins in HK-2 cell exposed to HG with or without transfection of Rap1b G12V and pre-treatment with CEBP-β siRNA or PGC-1-α siRNA. Values are means +/- SE. N=4, *P <0.01 versus 5 mM D-glucose; ¤P <0.01 versus 30 mM D-glucose; #P <0.01 versus 30 mM D-glucose + Rap1b G12V.
Figure S5. Schematic sketch of conceivable cellular events following exposure to HG ambience and overexpression of Rap1bG12V in renal tubular cells.

HG ambience led to an activation of Rap1 GAP and reduced Rap1 GTPase activity in renal tubular cells which inhibits ERK1/2 - C/EBP-β:PGC-1α nuclear translocation, and this in turn reduces the expression of mitochondrial antioxidant genes, e.g., Catalase and Mn-SOD as well as mitochondrial biogenesis genes, e.g., NRF1 and mtTFA. This eventually causes mitochondrial dysfunctions, including release of mitochondrial Cytochrome C, activation of caspases, fragmentation of DNA and cellular apoptosis. Conceivably, transfection of GTPase Rap1b interrupted the signaling pathway to ameliorate mitochondrial dysfunctions that occurs following HG ambience. In addition, overexpression of Rap1b possibly blocks the
phosphorylation and mitochondrial translocation of Drp1 protein that modulates mitochondrial dynamics and reduces the generation of ROS production and consequential cellular damage.
### 2. SUPPLEMENTAL TABLE  (Primer Sequence)

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**SUPPLEMENTAL DATA**

**mtDNA Studies:** The mtDNA damage to long and short DNA was monitored by PCR. Long-range PCR was employed to coamplify long and short mtDNA fragments. PCR products were subjected to 1.6% agarose gel electrophoresis followed by staining with ethidium bromide to detect 8636- and 316-bp DNA products. A ratio between the long and short DNA fragments was calculated by measuring the band intensity with a PhosphorImager (Molecular Dynamics, Sunny-vale, CA). For long PCR the primers were as follows: sense: 5’-AGTCATACGCAAAAGA-3’ and antisense: 5’-TCTAGAGGCTCTCAAG-3’. The primers for short PCR were as follows 5’-ATGCTCTGAGCTATGATC-3’ (sense) and 5’-GATTTGCGTAGGTTG-3’ (antisense).

**Assessment of mitochondrial transmembrane potential (∆ψm):** HK-2 cells were transfected with Rap1b, C/EBP-β-siRNA or PGC-1α-siRNA and plated on 35-mm glass-bottom culture dishes, and maintained at 37°C in a serum free medium for 12 hrs. Then they were treated with HG treatment for 3 days in a defined medium. Following which the cells were transferred to a phenol red-free DMEM (Life-Tech, Carlsbad, CA). 10 nM of TMRE dye (Molecular Probes) added to the medium and cells maintained at 37°C for 10 minimums. Following a brief wash with PBS the mitochondrial ∆ψm in intact cells was
assessed by FACS analyses. In addition, Confocal microscopy was performed using a wavelength of 582 nm, and the data from four different experiments were analyzed. In isolated mitochondria from renal tissues, the $\Delta \psi_m$ was gauged following a load of rhodamine 123 (Rh123) in a medium containing 150 mM sucrose, 5 mM MgCl$_2$, 5 mM KH$_2$PO$_4$, 20 mM potassium-HEPES, pH 7.4 in the presence or absence of 10 mM glutamate and 5 mM malate at 28°C. Afterwards, the ratio of fluorescence at 520-nm and 497-nm excitation wavelengths and 529-nm emission wavelength was calculated, as further detailed in previous publications (Biochica et Biophysica Acta. 850:436-448, 1986; Am J. Physiol Regul Integr Comp Physiol. 290:R1616-R1625, 2006).

**Mitochondrial enzyme activities:** Manganese superoxide dismutase (Mn-SOD) activity and CuZn-SOD activity was determined in mitochondrial fractions of kidney tissues using Superoxide Dismutase [SOD] Activity Assay Kit Alexis® Biochemicals, ABL-SOD-560-K101). Superoxide Dismutase [SOD] Activity Assay Kit not only measures total SOD activity but also can be used to distinguish CuZn-SOD or Mn-SOD activity separately. The mitochondrial catalase activity was measured using Catalase Activity Colorimetric/Fluorometric Assay Kit (BioVision, Inc). The levels of glutathione peroxidase (GSH-Px) were determined using the Glutathione Peroxidase (GSH-PX) Assay Kits (Biocompare, Inc). All assays above were performed by following the vendors’ guidelines.
Examination of fragmentation and length of long-axis of mitochondria: Kidney tissue blocks of ~1 mm³ size were diced from each kidney cortex for standard Electron Microscopy processing. Thin sections (0.5 um) were prepared from EPON embedded tissue blocks and examined by a transmission electron microscope operating at 60 KV and mitochondrial abnormalities delineated. To determine mitochondrial fragmentation several contiguous (side-by-side) digital images were generated. Percentage of cells that had less than 1% long filamentous mitochondria were reflective of mitochondrial fragmentation. To determine the length of long-axis of mitochondria, digital images were generated. Then, the length of individual mitochondria in a cell was measured by using NIH Image software (http://rsbweb.nih.gov/ij/). For each cell, approximately 25 mitochondria were measured to determine the percentage distribution of mitochondria with various lengths, either ranging 0-2 µm or >2 µm, and the latter measurement was considered to be representative of elongated filamentous mitochondria.

PGC-1α gene promoter analysis: Various deletion constructs of PGC-1α promoter were generated by PCR. The PCR products were cloned into Xhol- and Hind III-digested pSEAP2-Enhancer plasmid vector (CLONTECH) and confirmed by nucleotide sequencing. HK-2 cells were transfected with various plasmid deletion constructs using Lipofect AMINETM 2000 reagent. Minimal promoter activity of the PGC-1α promoter activity was measured in the
supernatants of the cell cultures using a Great EscAPE TM SEAP fluorescence detection kit (CLONTECH). The activities of various deletion constructs were expressed as the percentages of the activity in the deletion construct with the highest promoter activity, which was designated as being 100%.

Preparation of nuclear extracts and electrophoretic mobility assays (EMSA): Nuclear extracts used in EMSAs assays from HK-2 cell were performed as follows. Briefly, the nuclear extracts (10µg) were incubated with 1–3 µg/µl poly (dl-dC), 50 mM Tris-HCl, 750 mM KCl, 2.5mM EDTA, 0.5% Triton X-100, 62.5% glycerol (v/v), 1mM DTT) and 40,000 CPM of [γ-32P] dATP end-labeled oligonucleotides containing putative CRE (cAMP response elements) within the -146 to -132 bp region of the hPGC-1α promoter corresponding to GGCTGCCTTTGAGTGACGTCA CAC-3’. Next, the Rap1b G12V, Rap1b M17, mutant CRE site Oligo and C/EBP-β-siRNA and/or anti-PGC-1α were then transfected or added into HK-2 cells. The samples were then subjected to native 5% acrylamide gel electrophoresis for 1.5 hrs at 250 V. The gels were subsequently treated with acetic acid/methanol/water (10:30:60) for 15 min, dried and imaged using an instant imager.

Chromatin immunoprecipitation (ChIP) analysis: ChIP analysis was performed using a transcription factor ChIP kit according to the manufacturer’s instructions. Soluble chromatin was co-immunoprecipitated with anti-PGC-1α
antibody or an equal amount of control rabbit IgG. Co-precipitated DNA was analyzed by qPCR, using the primer pairs used for PCR analysis. The primer sequences spanning -2160 to -1938 region were as follows: Sense - 5'-GGCTTCTGTTTGCCTTGCTCAG-3' and antisense - 5'-ATACTGATACG CG ATTGTGA AGCG-3'. This region of amplification contains FoxO1-dependent binding element (FoxO1-DBE) in the Catalase enzyme promoter (NC-005102.2). The PCR was also performed for MnSOD with following sequences as: Sense - 5'-GTTCTCTTGCCTGACTGTT-3' and antisense 5'-CTGAA CCGTTTCCGTTGCTT-3'. The GAPDH promoter was also analyzed by ChIP in parallel as a control. Finally, the relative ChIP units were defined by the ratio of ChIP DNA to input DNA following densitometry.