Therapeutic Approach for Diabetic Nephropathy Using Gene Delivery of Translocase of Inner Mitochondrial Membrane 44 by Reducing Mitochondrial Superoxide Production

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Hyperglycemia-induced overproduction of mitochondrial reactive oxygen species has emerged as a major player in diabetic vascular complications. Mammalian translocase of inner mitochondrial membrane 44 (TIM44) was identified by upregulation in diabetic mouse kidneys. TIM44 functions as a membrane anchor of mitochondrial heat-shock protein 70 (mtHsp70) to TIM23 complex and is involved in the import of mitochondria-targeted preproteins into mitochondrial matrix. The process is dependent on inner membrane potential and ATP hydrolysis on ATPase domain of mitochondrial heat-shock protein 70. Hemagglutination virus of Japan–envelope vector that carries pcDNA3.1 plasmid that contains the full-length cDNA of TIM44 and control plasmid were injected weekly into the tail vein of uninephrectomized streptozotocin-induced diabetic CD-1 mice. The gene delivery alleviated proteinuria and renal hypertrophy at 8 wk after the injection, inhibited renal cell proliferation and apoptosis, and suppressed superoxide production. In vitro experiments, using human proximal tubular (HK2) cells, revealed that the gene delivery of TIM44 reversed high glucose–induced metabolic and cellular abnormalities such as enhanced reactive oxygen species production, increased ATP contents, alterations in inner membrane potential, increased cell proliferation, and apoptosis. Transfection with siRNA and expressing vector of TIM44 revealed that TIM44 facilitates import of antioxidative enzymes such as superoxide dismutase and glutathione peroxidase into mitochondria. The gene delivery of TIM44 therefore seems to be beneficial for the maintenance of mitochondrial function and is a novel therapeutic approach for diabetic nephropathy.


Diabetic nephropathy (DN) is one of the major complications and causes of death in patients with diabetes (1). Various mechanisms are implicated in the pathogenesis of DN, including increased aldose reductase activity (2), enhanced activity of protein kinase C isoforms (3,4), and increased formation of advanced glycation end products (5). Hyperglycemia-driven mitochondrial overproduction of reactive oxygen species (ROS) was identified as the underlying biochemical abnormality and is an emerging concept for the development of antivasculopathy principles in the treatment of diabetes (6,7). Hyperglycemia induces intracellular glucose oxidation, which generates NADH and pyruvate and enhances the influx of pyruvate into mitochondria. Pyruvate is oxidized by the tricarboxylic acid cycle to produce NADH and FADH2, which flow through the mitochondrial electron transport chain that is formed by inner membrane–associated enzyme complexes. Electron transfer through complexes I, III, and IV generates a proton gradient; however, when electrochemical potential difference is high under high glucose (HG) condition, ROS is generated at complex I and the interface between ubiquinone and complex III. The inhibitor of complex II, manganese superoxide dismutase (Mn-SOD), and uncouple protein-1 prevent the HG–induced ROS production in bovine endothelial cells and inhibits the subsequent activation of protein kinase C, formation of advanced glycation end products, sorbitol accumulation, and NF-κB activation (7,8). ROS are considered as important mediators for several biologic responses, including cellular proliferation, extracellular matrix deposition, and apoptosis (9).

A major site for the ROS production is mitochondria, and it plays an important role in diabetic vascular complications (7,8). Overexpression of catalase targeted to mitochondria reduced ROS production and resulted in increased life span, supporting the free radical theory of aging and the importance of mitochondria as a source of ROS (10). Mitochondria consist of approximately 1000 different proteins, and most of them are encoded by the nuclear genome, which consists of approxi-
mately 10 to 20% of cellular proteins (11). The vast majority of mitochondrial proteins are synthesized in the cytosol and imported into mitochondria by protein machineries that are located in the mitochondrial outer and inner membranes. Preproteins use a common translocase of the outer mitochondrial membranes (TOM) complex, whereas there are two distinct translocase of inner mitochondrial membrane (TIM) complexes, TIM23 and TIM22 complexes, which are involved in translocation across the inner membrane of matrix protein with integral signals, respectively (12). TIM23 translocase is formed by a 90-kD complex that consists of two essential proteins, TIM23 and TIM17, which form channels for translocation of preproteins. Protein translocation across the inner mitochondrial membrane requires two driving forces: An inner membrane potential (∆ψ) and an ATP-dependent import motor, which consists of mitochondrial heat-shock protein 70 (mtHsp70), the translocase subunit mammalian TIM44, and the co-chaperone Mge1. The mtHsp70 binds to the extended segment of preproteins that emerges on the matrix side of the TIM23 channel powered by binding and hydrolysis of ATP. TIM44 functions as a membrane anchor for the ATPase domain of mtHsp70, whereas Mge1 is a nucleotide-exchange factor that promotes the reaction cycle or rebinding of ATP to mtHsp70 (13,14).

TIM44 first was identified in streptozotocin (STZ)-induced diabetic mouse kidney during the PCR-based differential screening analyses (15–17). Upregulation of TIM44 as a response to elevated blood glucose may lead to efficient import of nuclear encoded mitochondrial matrix proteins that are required for maintenance of mitochondrial functions. It was reported that hyperglycemia also leads to reduction in antioxidative defense, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (18,19). In our previous study, we confirmed that the gene delivery of TIM44 into a balloon injury model of diabetic rats ameliorated neointimal proliferations by reducing ROS production, inflammatory responses, and cell proliferation in carotid artery (20). Here we hypothesized that TIM44 may have a protective role in DN by importing various mitochondrial matrix proteins, especially antioxidative defense enzymes such as SOD and glutathione peroxidase. We used the uninephrectomized STZ-induced diabetes mouse model to investigate the therapeutic potential of gene delivery of TIM44 against DN and its possible mechanisms.

Materials and Methods
Animal Model of Diabetes and Experimental Design

Twelve-week-old male CD-1 mice (Charles River Co., Yokohama, Japan) were uninephrectomized and divided into three groups: Uninephrectomized nondiabetic group (NX), diabetic group treated with pcDNA 3.1 plasmid (STZ, Invitrogen, Carlsbad, CA), and diabetic group treated with pcDNA 3.1/TIM44 plasmid (TIM44; n = 18 per group). Two weeks after uninephrectomization, STZ and TIM44 groups were received intravenous injections of a single dose of 150 mg/kg STZ (Sigma, St. Louis, MO) in citrate buffer at pH 4.6. Hyperglycemia (>15 mmol/L) was confirmed 3 d after STZ administration. Hemagglutination virus of Japan (HVJ)-envelope vector (GenomeONE-Neo, Ishihara Sangyo, Osaka, Japan) that carried plasmid DNA was injected into the tail vein weekly after diabetes induction. Mice were killed at weeks 1, 2, and 8, and urine and blood samples were collected. Kidney tissues were removed and subjected to the following experiments. All animal procedures were in accordance with the Guide for Care and Use of Laboratory Animals at Okayama University, Advanced Science Research Center, Department of Animal Resources.

Generation of pcDNA3.1/TIM44 and Gene Transfer

Expression of TIM44 (accession no. U69898) was carried out using pcDNA3.1/V5-His TOPO Expression Kit (Invitrogen). cDNA that contained a full-coding region without stop codon were generated by reverse transcription–PCR and subcloned into pcDNA3.1/V5-His TOPO to prepare TIM44 (pcDNA3.1/TIM44). Primers were 5'-AACAT-GCCGCAGCAGCTCT-3' and 5'-GAGGATCTGCTCTGTGCTGG-3'. Diabetic animals were treated weekly with 80 μg of plasmid DNA/ mouse carried by 8 AU of HVJ-E vector in 120 μl of suspension buffer by tail-vein injection according to the manufacturer’s protocol. One week after the gene delivery of pcDNA3.1 + V5/His/LacZ plasmid (Invitrogen), kidney tissues were subjected to β-galactosidase (β-Gal) staining for evaluation of the efficiency of gene transfer (n = 6).

Light Microscopy, Immunohistochemistry, and Transferase (Tdt)-Mediated dUTP Nick-End Labeling

Kneys were fixed in 10% formaldehyde and embedded in paraffin, and 2- and 4-μm sections were prepared. Two-micrometer sections were stained with periodic acid-Schiff, and the measurement of glomerular and tubular diameter was carried out using Zeiss LSM image browser software. Four-micrometer sections were deparaffinized and rehydrated, and endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide and methanol. Sections were pretreated by microwave for 20 min in citrate buffer for antigen retrieval. PCNA (Santa Cruz Biotechnology, Santa Cruz, CA) immunochemistry staining was performed with VECTASTAIN Universal Quick kit (Vector Laboratories, Burlingame, CA). Investigation of apoptotic cells was performed using terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP nick-end labeling (TUNEL) with in situ Apoptosis Detection Kit (Chemicon International, Temecula, CA). The numbers of TUNEL-positive cells were counted in 50 glomeruli and in 104 μm2 tubulointerstitial area.

Oxidant Fluorescence Microtopography

Intracellular generation of O2·− was assessed using hydroethidine. O2·− reacts with hydroethidine to produce ethidium bromide, which binds to nuclear DNA and gives red fluorescence color. Unfixed frozen kidneys were cut into 30-μm-thick sections and placed on a glass slide. Hydroethidine (2 × 10−6 mol/L in PBS) was applied to each tissue section and coverslipped. Slides were incubated in a light-protected humidified chamber at 37°C for 30 min. Images were obtained with a confocal laser scanning microscope (Carl Zeiss Laser Scanning System LSM 510; Carl Zeiss, Jena, Germany) (21,22). Fluorescence was detected with a 585-nm long-pass filter. All tissues were processed and imaged in parallel. Laser settings were identical for acquisition of images from STZ, TIM44, and NX control specimens.

In Vitro Experiments

HK2 cells were purchased from Kurabo (Osaka, Japan). HK2 cells were grown in keratinocyte-serum free medium (Life Technologies) in the presence of 5 ng/ml recombinant EGF and 0.05 mg/ml bovine pituitary extract. A total of 1 × 105 cells per well in 96-well culture plates or 5 × 105 cells per well in 24-well culture plates or 2 × 106 Lab-Tek16 chamber slides (Nalge Nunc International, Rochester, NY)
HK2 cells were incubated with DMEM that contained normal glucose with DMEM that contained HG (300 mg/dl) for 6 to 24 h. As controls, HK2 cells were incubated with DMEM that contained normal glucose (100 mg/dl).

Cell Proliferation and Apoptosis Assay
HK2 cells in 96-well plates were subjected to cell proliferation ELISA using 5-bromo-2′-deoxyuridine (BrdU; Roche, Penzburg, Germany). BrdU labeling solution was added to culture media and reincubated for an additional 6 h. The culture media were removed and serially incubated with FixDenat, anti–BrdU-POD, washing solution, and substrate solution. Finally, we measured the absorbance of the samples in an ELISA plate reader (BioRad, Hercules, CA) at 450 nm (reference wavelength approximately 492 nm). Cell proliferation also was detected by immunocytochemistry using PCNA antibody. Apoptosis of HK2 cells was evaluated with in situ Apoptosis Detection Kit.

Intracellular ROS
Cells in 96-well plates were loaded with 10 μM CM-H2 DCFDA (5-[and -6]-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate, acetyl ester; Molecular Probes, Eugene, OR), incubated for 1 h at 37°C, and analyzed with an HTS 7000 Bio Assay Fluorescence Plate Reader (Perkin Elmer, Norwalk, CT). ROS production was determined from an H2O2 standard curve (7).

ATP Determination
A total of 1 × 10^5 HK2 cells in 24-well culture plates were collected; lysed with 20 μl of 1× Passive Lysis Buffer (Promega, Madison, WI); reconstituted in 200 μl of ATP determination reaction mix that contained 1 mM dithiothreitol, 0.5 mM luciferin, and 1.25 μg/ml luciferase; and subjected to a luminometer measurement (AB-2200; ATTO, Tokyo, Japan).

Δψ Integrity Measurement
To measure Δψ, the ApoAlert membrane sensor Kit (Clontech, Palo Alto, CA) was used (23,24). We added the Mitosensor fluorescence reagent directly into HK2 cells in 96-well culture plates and chamber slides. Fluorescence was measured with an multidetection microplate reader (Synergy HT; Bio-Tek Instruments, Winooski, VT) using the Rhodamine channel to measure the red fluorescence produced when the Mitosensor reagent is taken up in the mitochondria, where it forms aggregates that exhibit red fluorescence. If Δψ is altered, then the Mitosensor reagent remains in monomeric form in the cytoplasm and shows green color. Mitosensor Fluorescence micrographic images were obtained with a confocal laser scanning microscope (Zeiss).

Mitochondrial Import of Mn-SOD and Glutathione Peroxidase in HK2 Cells Transfected with pcDNA3.1/TIM44 and siRNA
After HK2 cells were transfected with pcDNA3.1, pcDNA3.1/TIM44, and TIM44 siRNA and stimulated with HG, mitochondria fractions were isolated with ApoAlert Cell Fraction Kit (Clontech). The preparation of mitochondria fraction was assayed with Western blot analysis using Cytochrome C antibody supplied in the kit. Ten micrograms of protein was loaded onto SDS-PAGE, and Western blot analyses using goat anti-human polyclonal Mn-SOD (Santa Cruz Biotechnology), sheep polyclonal anti-human glutathione peroxidase (Abcam, Cambridge, UK), and goat polyclonal anti-human TIM44 (Santa Cruz) were performed.

Statistical Analyses
Data are presented as mean ± SD. Statistical analysis was performed by unpaired t test or a one-way ANOVA by Fisher t test when multiple comparisons against the control were required. P < 0.05 was considered significant. The data were analyzed with Dr. SPSS II for Windows release 11.0.1] (SPSS, INC., Chicago, IL).

Results
Gene Delivery to Mouse Kidney Using HVJ-E Vector
The efficiency of gene transfer was tested by pcDNA3.1/LacZ transfection and β-Gal staining. As shown in Figure 1A, intense staining was seen in kidney cortex after plasmid injection from tail vein; β-Gal activity was visualized in both glomeruli and tubules. In Figure 1B, TIM44 protein expression in the TIM44 group was approximately 2.5-fold greater than that of the STZ group. In the STZ group, endogenous TIM44 protein expression was detected in glomeruli and interstitial cells, whereas the signals were weak in tubules. After gene transfer of TIM44, TIM44 protein was highly expressed in tubules, predominantly in proximal tubules, and higher expression in glomeruli also was observed from week 1 to week 8 (Figure 1C).

Gene Delivery of TIM44 Alleviates Proteinuria and Renal Hypertrophy
At week 8, urinary albumin/creatinine ratio was approximately four-fold higher in the STZ group than that of the NX group, and TIM44 treatment significantly reduced proteinuria (Figure 2A). The kidney/body weight ratio was increased in the STZ group compared with that of the NX group; TIM44 treatment significantly reduced the kidney weight and inhibited renal hypertrophy (Figure 2B). We further investigated the protective effects of TIM44 on DN by histopathology assessment at week 8. Diabetic mice in the STZ group revealed significant tubular and glomerular hypertrophy as well as expanded mesangial areas compared with the NX group; the treatment with TIM44 was associated with a significant reduction in renal hypertrophy and mesangial areas (Figure 2C). In addition, the glomerular and tubular diameters and cell numbers significantly increased in the STZ group compared with those of the NX control mice (Figure 2, D through G), whereas the treatment with TIM44 reversed these changes. From these results, TIM44 gene delivery significantly inhibited glomerular and tubular hypertrophy in diabetic kidney from week 1 to week 8 and alleviated proteinuria at week 8.

Gene Delivery of TIM44 Inhibits Renal Cell Proliferation and Apoptosis in Diabetic Mice
We evaluated the proliferation of kidney cells with PCNA staining. PCNA-positive cells were seen occasionally in the NX group, and they increased significantly in tubules as well as in glomeruli in the STZ group at weeks 1 and 2. The gene delivery of TIM44 reduced PCNA-positive cells (Figure 3, A through C). At week 8, PCNA-positive cells returned to basal levels in the STZ and TIM44 groups, and there were no differences among
Figure 1. Gene transfer of translocase of inner mitochondrial membrane 44 (TIM44) into uninephrectomized diabetic CD-1 mice. (A) β-Galactosidase (β-Gal) staining shows activity of β-Gal indicated by blue color in glomeruli and tubules after intravenous injection of pcDNA3.1/LacZ. (B) Western blot demonstrates TIM44 protein expression in the kidneys at different time points after injection of control plasmid vector or pcDNA3.1/TIM44, respectively. Whole-kidney extracts were separated on SDS and immunoblotted with a specific mAb against TIM44. Compared with those that were treated with pcDNA plasmid (streptozotocin [STZ]-induced diabetic group [STZ group]), animals that were transfected by pcDNA3.1/TIM44 (TIM44 group) show high expression of TIM44 in the kidneys. (C) Immunohistochemistry staining shows the localization of TIM44 protein in kidneys after the gene transfer. Weak staining was observed in the kidneys of the STZ group; marked TIM44 expression is observed in both glomeruli and tubules in the TIM44 group (*P < 0.01 versus STZ group). Bars = 100 μm (C). n = 6 mice per group.
Figure 2. The effects of gene transfer of TIM44 on diabetic nephropathy (DN) in diabetic mice. (A) Urinary albumin/creatinine ratios in nondiabetic uninephrectomized (NX) and uninephrectomized STZ-induced diabetic mice that were treated with pcDNA3.1 (STZ) and treated with pcDNA3.1/TIM44 (TIM44). (B) Kidney hypertrophy in STZ mice is ameliorated by the treatment of TIM44. (C through G) Periodic acid-Schiff staining of kidney tissues shows glomerular and tubular hypertrophy, and mesangial matrix accumulation in STZ group 8 wk after injection. The treatment of TIM44 reverses such pathologic changes (*P < 0.05, **P < 0.01 versus NX group; #P < 0.05, ###P < 0.01 versus STZ group). Bars = 100 μm (C). n = 6 mice per group.
Figure 3. Cell proliferation and apoptosis in diabetic mouse kidneys. (A) Immunoperoxidase staining with proliferation marker PCNA in the kidneys of NX mice, STZ mice, and TIM44 mice. PCNA-positive cells in kidneys of the STZ group dramatically increase at weeks 1 and 2, and pcDNA/TIM44 treatment significantly reduces PCNA-positive cells in both glomeruli and tubules. (B and C) Proliferating cells are barely seen in all three groups at week 8. (D through F) By in situ transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay, apoptotic cells are observed predominantly in tubules in the STZ group at week 8 (D); the number of apoptotic cells is significantly reduced by pcDNA/TIM44 treatment (F). The condensed chromosome (arrow) is seen in proximal tubule of diabetic kidney by electron microscopy (E) (*P < 0.01 versus NX group; **P < 0.01 versus STZ group). Bars = 100 μm (A and D) and 10 μm (E). n = 6 mice per group.
the three groups. No apparent apoptotic cells were seen in the NX group during the entire observation period; apoptotic cells were clustered in tubules in the STZ group at week 8 (Figure 3D); the condensed chromatin in apoptotic cells were observed in proximal tubules by electron microscopy (Figure 3E). The apoptotic cells were not observed in podocytes and other glomerular cells by electron microscopy. The treatment of pcDNA3.1/TIM44 significantly reduced the number of apoptotic cells in tubular cells (Figure 3F).

**Gene Delivery of TIM44 Inhibits Renal Superoxide Production**

Kidney tissues were labeled with hydroethidine, which produces a red fluorescence when oxidized to ethidium bromide under the presence of O$_2^-$; Diabetic mice kidneys in the STZ group showed a significant increase in hydroethidine fluorescence when compared with the NX group; the gene delivery of TIM44 markedly decreased renal cell–derived superoxide (Figure 4).

**Gene Delivery of TIM44 Normalizes HG-Induced Cell Proliferation and Apoptosis**

HK2 cells were subjected to cell proliferation and apoptosis assay. HG-induced cell proliferation was demonstrated by BrdU ELISA assay and PCNA staining. Transfection of TIM44 into HK2 cells significantly inhibited proliferation, whereas cells that were transfected with TIM44 siRNA slightly increased their proliferation (Figure 5, A through C). The effects of TIM44 on apoptosis were evaluated by TUNEL staining. Apoptotic cells were seen occasionally in HK2 cells in the normal glucose group (2 ± 0.5%), whereas the number of apoptotic cells significantly increased in the HG group (11 ± 0.2%) and HG + pcDNA groups (11.5 ± 1.0%). Apoptotic cell numbers seemed to decrease in the TIM44-treated group (HG + TIM44; 5.0 ± 1.0%) and increase in the TIM44 siRNA treated group (HG + siRNA; 15.0 ± 1.3%; Figure 5, D and E).

**TIM44 Gene Delivery Facilitates Import of Mn-SOD and Glutathione Reductase**

We isolated mitochondrial fractions and performed Western blotting to investigate the import of Mn-SOD, glutathione peroxidase, and TIM44 protein into mitochondria. Cytochrome c is an inner mitochondria protein, and the import is TIM44 independent; the amount of cytochrome c in mitochondria was not altered by the treatment of TIM44 and TIM44 siRNA. In contrast, Mn-SOD and glutathione peroxidase are mitochondrial matrix protein, and their import process is TIM44 dependent. TIM44 transfection significantly increased TIM44 protein expression and facilitated the mitochondria matrix proteins such as Mn-SOD and glutathione peroxidase, whereas TIM44 siRNA inhibited TIM44 protein and reduced import of Mn-SOD and glutathione peroxidase into mitochondria (Figure 6).

**Gene Delivery of TIM44 Normalizes HG-Induced ROS Production, ATP Synthesis, and Δψ Alteration In Vitro**

ROS production increased at 6 h after HG stimulation, and it maintained for 24 h (Figure 7A). The gene delivery of TIM44 significantly suppressed ROS production at all time points. ATP levels increased at 6 h after HG incubation, then decreased to the control levels at 12 h, followed by a slight decrease at 24 h (Figure 7B). The gene delivery of TIM44 significantly reduced ATP contents at 6 h. The change of Δψ was investigated by qualification of fluorescence intensity using rhodamine and FITC channels with confocal microscopy and fluorescence plate reader. Mitosensor exhibits intense red fluorescence, indicating that Mitosensor is taken up by the mitochondria, where it forms aggregates. In the HG condition, an initial hyperpolarization was revealed by an increase of red and a decrease of green color at 6 h. This was followed by a depolarization, which was revealed by an increase in green color at 12 and 24 h. The time course of the inner membrane hyperpolarization and depolarization process corresponded to the change in ATP levels. The gene delivery of TIM44 partially but significantly reversed HG-induced alteration of Δψ (Figure 7, C, D, and E).

**Discussion**

Mitochondria includes approximately 1000 different polypeptides, most of which are encoded by the nuclear genome, synthesized by the cytoplasmic ribosomes, and imported into mitochondria, whereas mitochondrial genome encodes only 37 genes. Import into mitochondria is mediated by transport machinery in the mitochondrial membranes (25). Three mitochondrial transport machinery complexes have been identified: The TOM complex in the outer membrane and the TIM23.
and TIM22 complexes in the inner membrane (26,27). These machinery complexes mediate protein translocation across or into the mitochondrial membranes and guide the proteins to their destinations within mitochondria. The TIM23 complex consists of two integral membrane proteins, TIM23 and TIM17, and a peripheral membrane protein, TIM44 (12). Two models for the mechanism of protein import into mitochondrial matrix by TIM23 complex have been proposed: The “Brownian ratchet” and “power stroke” models (11). In the latter, TIM44 functions as a membrane anchor of mtHsp70 to TIM23 complex and ATP hydrolysis confined to the ATPase domain of mtHsp70, which induces conformational change of mtHsp70 and generates a pulling force on the preprotein and leads to the release of mtHsp70 from TIM44 (28–30). Interaction of Mge1 with the preprotein-bound mtHsp70-ADP triggers ADP dissociation and rebinding of ATP to nucleotide-free mtHsp70, inducing the opening of the substrate-binding pocket and release of mtHsp70 (13). Therefore, mitochondrial matrix protein import via TIM23 is dependent on inner membrane potential as well as ATP hydrolysis. TIM44 was detected in mitochondrial inner membrane fraction as well as in the matrix fraction (31–33). The localization on inner membrane suggested that mammalian TIM44 involves in the power stroke model in preprotein import (33). Available data suggest that the mitochondrial im-

Figure 5. Cell proliferation and apoptosis in human proximal tubular cells (HK2) cultured under high glucose (HG) condition. HK2 cells were cultured in DMEM that contained normal glucose (NG; 100 mg/dl D-glucose) and HG (300 mg/dl D-glucose). HK2 cells under the HG condition were transfected with pcDNA3.1 (HG + pcDNA), pcDNA3.1/TIM44 (HG + TIM44), and TIM44 siRNA (HG + TIM siRNA) using Lipofectamine 2000 reagent. Cell proliferation was revealed by PCNA staining and 5-bromo-2'-deoxyuridine ELISA assay, and apoptosis was assessed by TUNEL. Transfection of pcDNA3.1/TIM44 into HK2 cells significantly inhibits HG-induced cell proliferation (A, B, and C) and apoptosis (D and E), whereas TIM44 siRNA treatment increases cell proliferation and apoptosis (*P < 0.05, **P < 0.01). Six independent experiments were repeated.
Due to a hemodynamic effect, proteinuria may improve of histology. Because the early proteinuria in DN is due to a hemodynamic effect, i.e., glomerular hyperfiltration, it may be less sensitive to the mitochondria-targeted therapies, and the inhibition of proteinuria was relatively small at week 8. The limitation of this study is the quantification of intracellular O$_{2}^{-}$ by the use of hydroethidine as an intracellular probe. Although this is the most frequently used assay, hydroethidine can be oxidized by H$_{2}$O$_{2}$/redox-active metal ions, peroxynitrite, and cytochrome c other than O$_{2}^{-}$ and therefore may overestimate the ROS production (35).

In these experiments, we can raise the possibility that the gene delivery of TIM44 may be protective against the direct toxicity of STZ, which is a potent inducer of ROS production and apoptosis/proliferation. Although further investigation using spontaneous diabetes models is required, the in vitro studies using HK2 cells indicated that TIM44 gene delivery reversed the HG-induced metabolic and cellular abnormalities. Another criticism is that proximal tubular cells in vivo should not at least exhibit glucose-driven increases of mitochondrial substrate metabolism because of lower glycolytic activity. In proximal tubular cells, glucose undergoes oxidative metabolism and the lactate production is very low, i.e., the activity of glycolysis is low and more glyconeogenic (36). However, the activities of glycolytic enzymes are significantly higher in diabetic kidney (37). It is not conclusive that glycolytic enzyme activity changes that are measured in the whole cortex are in the proximal tubules; however, the possibility of such notion was raised by in vitro observation. In primary cultures of proximal tubular cells, the activities of key enzymes of glyconeogenic pathway decrease, together with an increase in the rate of glycolysis and glycolytic enzyme activities (38,39). Therefore, the use of HK2 cells in culture may be an appropriate and representative model for DN in vivo.

Mitochondria play a central role in a variety of cellular processes, including proliferation and apoptosis. In mice that expressed a proofreading-deficient version of the mitochondrial DNA polymerase $\gamma$, mitochondrial DNA mutations accumulated and displayed accelerated aging. In these mice, accumulation of mitochondrial DNA mutations was not associated with increased oxidative stress but was correlated with the induction of apoptosis (40). In our experiments, the cell proliferation and apoptosis are enhanced in diabetic mice kidneys and HK2 cells that were treated with the HG condition, whereas the overexpression of TIM44 reduced apoptosis and inhibited cell proliferation. Because mitochondrial dysfunction leads to the mitochondrial outer membrane permeabilization, release of cytochrome c, and the initiation of mitochondrial pathway of apoptosis, the maintenance of mitochondrial function by TIM44 may be beneficial for the amelioration of HG-induced apoptosis.

For normalization of mitochondrial ROS production in diabetic states, an uncoupler of oxidative phosphorylation by uncouple protein-1, Mn-SOD, and an inhibitor of electron transport chain complex II has been reported (7). We would like to add TIM44 as one of therapeutic targets to sustain mitochondrial function and normalize ROS production in the diabetic state by facilitating the mitochondrial matrix protein import. These results indicate that the application of small molecules that enhance the activities of TIM44 and related import machinery molecules may exhibit therapeutic potentials in DN as well.
Figure 7. Reactive oxygen species (ROS) production, ATP levels, and mitochondrial membrane potential (∆ψ) in HK2 cells that were cultured under the HG condition. HK2 cells were cultured in DMEM as described in Figure 5. (A) Intracellular ROS measured with CM-H2DCFDA. The delivery of pcDNA3.1/TIM44 significantly suppresses ROS production compared with pcDNA3.1 plasmid–treated HK2 cells, whereas TIM44 siRNA increases ROS production. (B) Time-course changes in cellular ATP content. The treatment of pcDNA3.1/TIM44 significantly reverses the increase in ATP content at 6 h after HG stimulation, whereas TIM44 siRNA treatment shows a further increase compared with HG + pcDNA. (C and D) For measurement of ∆ψ, the ApoAlert membrane sensor Kit (Clontech) was used. If ∆ψ is not altered, then Mitosensor is taken up in the mitochondria, where it forms aggregates that exhibit red fluorescence. Mitosensor reagent remains in monomeric form showing green color in the cytoplasm because of depolarization of ∆ψ. An initial hyperpolarization is revealed by increase of red fluorescence at 6 h after HG stimulation, which is followed by a depolarization at 12 and 24 h. The gene delivery of TIM44 significantly reversed HG-induced alteration of ∆ψ (*P < 0.01, HG + pcDNA versus HG + TIM44). Six independent experiments were repeated.
as other microvascular complications, such as neuropathy and retinopathy.

**Conclusion**

The gene delivery of TIM44 into diabetic mice alleviated renal hypertrophy and inhibited renal cell proliferation and apoptosis by reducing ROS production. *In vitro* experiment revealed that the gene delivery of TIM44 normalized HG-induced enhanced ROS production and increased ATP production and alteration in inner membrane potential, cell proliferation, and apoptosis. Treatment with siRNA and expression vector for TIM44 clarified that import of antioxidative enzymes such as SOD and glutathione peroxidase was facilitated by TIM44. These observations suggest that TIM44 is a novel target in the therapeutic interventions for DN; TIM44 gene delivery may provide a new avenue for exploring a novel therapeutic strategy for DN.

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