Mitochondria-targeted peptide SS-31 attenuates renal injury via an antioxidant effect in diabetic nephropathy

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Among the various types of diabetes complications, diabetic nephropathy (DN) is the most common renal complication and is a leading cause of end-stage renal disease. Early alterations in DN include glomerular hyperfiltration, glomerular and tubular epithelial hypertrophy, and the development of microalbuminuria, followed by the development of glomerular basement membrane thickening, the accumulation of mesangial matrix, cell apoptosis and overt proteinuria, eventually leading to glomerulosclerosis and end-stage renal disease (5, 30). Despite the available modern therapies of glycemic and blood pressure control, many patients continue to show progressive renal damage (45). Therefore, it is extremely important to identify novel interventions to halt the progression of DN.

It is increasingly evident that changes in cellular function resulting in oxidative stress play a key role in the development and progression of DN (11). Free radicals such as superoxide can induce cell and tissue injuries through lipid peroxidation, activation of NF-κB, production of peroxynitrite, PKC activation, and induction of apoptosis (17). The generation of reactive oxygen species (ROS), specifically O_2^{-•}, by damaged or dysfunctional mitochondria, has been postulated as the primary initiating event in the development of diabetes complications (35). High glucose (19), advanced glycation end products (7), ANG II (23), and transforming-growth factor (TGF)-β1 (39) all increase intracellular ROS in renal cells and contribute to the development and progression of diabetic renal injury. Interestingly, oxidative stress has been suggested as a common product of much of mechanisms that are involved in pathogenesis of DN (48). In fact, in the tangled web of DN pathogenesis, oxidative stress activates other pathogenic pathways, other pathways cause injury via oxidative stress, and oxidative stress directly leads to injury (48). Thus, inhibition of oxidative stress may constitute a focal point for multiple therapeutic synergies.

A major site for the ROS production is the mitochondria, and they play an important role in diabetic vascular complications (35). 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 (42). Mitochondrial superoxide has been shown to play a pivotal role in mediating mitochondrial damage that leads to renal injury during hyperglycemia (33). Reduction of mitochondrial ROS generated during hyperglycemia has been shown to prevent renal damage in vivo and in vitro (4, 52). Therefore, novel approaches to block mitochondrial ROS generation may offer a therapeutic benefit by preventing the development of diabetic renal disease.

SS-31 peptide (D-Arg-2\(^{6}\), 6’-dimethyltyrosine-Lys-Phe-NH\(_2\)) belongs to a family of small cell-permeable peptides that target and concentrate in the inner mitochondrial membrane, the site of ROS generation (55). This peptide can scavenge ROS and reduce mitochondrial ROS production and, thereby, prevent mitochondrial permeability transition and cytchrome c (Cyt c) release (55). SS-31 has several advantages, including being a small water-soluble peptide, being able to target and accumulate at the inner mitochondrial membrane in a potential-independent manner, and being able to protect against mitochondrial depolarization (49). SS-31 has been shown to readily...
SS-31 ATTENUATES RENAL INJURY

Materials and Methods

Materials. Chemical reagents were obtained from Sigma (St. Louis, MO). SS-31 was provided by ChinaPeptides (Shanghai, China). Collagen IV (sc-18178), fibronectin (sc-6952), and Cyt c (sc-7159) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Nox4 (ab133303), Bax (ab7977), Bcl-2 (ab32124), and thioreredoxin-interacting protein (TXNIP; ab188865) antibodies were obtained from Abcam (Cambridge, UK). Antibodies for cleaved caspase-3 (9664), caspase-3 (9662), p-p38 MAPK (9215), p38 MAPK (9212), p-CREB (9198), and CREB (9197) were purchased from Cell Signaling Technology (Beverly, MA). TGF-β1 (18978-1-AP) and TRX2 (13089-1-AP) antibodies were obtained from Proteintech (Chicago, IL). TRZol reagent was obtained from Invitrogen Life Technologies (Carlsbad, CA). DeadEnd Fluorometric TUNEL System and the reverse transcription system were obtained from Promega (Madison, WI). SYBR Premix Ex TaqTM II was purchased from Takara (Shiga, Japan).

Animal model. Studies were performed in male CD-1 mice (Charles River Laboratories, Vitalriver, Beijing, China), weighing 18–22 g. Mice were housed in the animal facilities of the Hebei Medical University with free access to food and water. The animals underwent uninephrectomy and were randomly divided into four groups (n = 8): nondiabetic control group (N), nondiabetic group administered SS-31 (N + SS-31), a diabetic group administered saline (STZ), and a diabetic group administered SS-31 (STZ + SS-31). Mice received an intraperitoneal injection of STZ (Sigma) at 150 mg/kg body wt. Hyperglycemia (>16.7 mmol/l) was confirmed 3 days after STZ administration. N + SS-31 Group and STZ + SS-31 Group mice received a daily intraperitoneal injection of SS-31 at 3 mg/kg body wt, and STZ Group mice received an injection of saline in an identical manner. At 8 wk after STZ, all of the animals were killed. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Hebei Medical University.

Measurements of urine 8-hydroxydeoxyguanosine by ELISA. Urine specimens were centrifuged at 1,500 rpm for 10 min to remove particulates. The supernatants were used, and 8-hydroxy-2-deoxyguanosine (8-OHdG) levels were measured by using a competitive in vitro ELISA kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer’s directions.

Light microscopy, immunohistochemistry, and transferase terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling analysis. Kidneys were fixed in 4% paraformaldehyde overnight, embedded in paraffin. Two- and four-micrometer sections were prepared. Two-micrometer sections were stained with periodic acid–Schiff (PAS), and 35–50 glomeruli per kidney were examined in a blinded manner. A semiquantitative index was used to evaluate the degree of glomerular mesangial expansion and sclerosis, as previously described (53). Each glomerulus on a single section was graded from 0 to 4, with 0 representing no lesion, and 1, 2, 3, and 4 representing mesangial matrix expansion or sclerosis, involving ≤25, 25 to 50, 50 to 75, or >75% of the glomerular tuft area, respectively. Mean glomerular tuft volume (GV) was determined from the mean glomerular cross-sectional tuft area (GA) as described previously (10). GV was calculated as GV = β/k × (GA)3/2, with β = 1.38, the shape coefficient for spheres, and k = 1.1, a size distribution coefficient. GA was measured using HPIAS-2000 image analysis software (Champion Image, Wuhan, China).

Kidney sections (4 μm) were deparaffinized with xylene and rehydrated in graded ethanol. Endogenous horseradish peroxidase activity was blocked with 3% H2O2 in 100% methanol for 30 min. Antigen retrieval was performed by microwave treatment in 10 mM citrate buffer for 15 min. Sections were incubated with primary antibodies for fibronectin (1:50 dilution), collagen IV (1:50 dilution), Bax (1:200 dilution), Bcl-2 (1:200 dilution), respectively, overnight at 4°C. Sections were then washed and incubated with biotinylated secondary antibody and horseradish peroxidase-conjugated streptavidin. Labeling was visualized with 3,3-diaminobenzidine to produce a brown color, and sections were counterstained with hematoxylin.

Fig. 1. SS-31 alleviates proteinuria, renal hypertrophy and urinary 8-hydroxy-2-deoxyguanosine (8-OHdG) excretion in diabetic mice. A: blood glucose concentration (8 wk). B: 24-h urinary albumin excretion (UAЕ). C: kidney/body wt ratio. D: 24-h urinary 8-OHdG excretion. All values are expressed as means ± SE. **P < 0.01 vs. nondiabetic (N) Group. #P < 0.05 vs. streptozotocin-induced diabetes plus saline (STZ) Group.
Investigation of apoptotic cells was performed using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) with DeadEnd Fluorometric TUNEL System, according to the manufacturer’s instructions. The numbers of TUNEL-positive apoptotic cells were counted from 10 different fields (×400) for each sample and were averaged.

Cell culture. Mouse mesangial cells (MMCs; no. CRL-1927) were obtained from American Type Culture Collection (Manassas, VA). They were cultured in DMEM-F12 medium (3:1) that was supplemented with 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin in a 95% air-5% CO₂ atmosphere. MMCs were grown to 75–85% confluence, washed once with serum-free DMEM-F12 medium, and then growth-arrested in serum-free DMEM-F12 medium for 24 h to synchronize the cell growth. After this time period, cells were stimulated with normal glucose (NG; 5.6 mM), HG (30 mM), NG plus mannitol (24.4 mM) as an osmotic control, NG plus mannitol plus SS-31 (100 nM), HG plus SS-31 (100 nM), and HG plus SB203580 (10 µM; Promega) at indicated time points.

Real-time PCR. Total RNA and then cDNA were prepared from kidney cortex tissues and cultured cells using TRIzol reagent and RT-PCR kits (Promega). The primers used were Bax, sense 5′-CT-GGATCCAAGACCAAGGGTG-3′ and antisense 5′-GTGAAGACTCTCACCCACAAA-3′; Bcl-2, sense 5′-GAACTGGGGAGGTGATTGTTG-3′ and antisense 5′-GCATGCTGGGGCCATATAGT-3′; TXNIP, sense 5′-CTCCTGTTGCATAGAAGGGTCT-3′ and antisense 5′-CATCTCGTTTCACCTGCTG-3′; TGF-β1, sense 5′-AGGGCTACCTGAGCATAC-3′ and antisense 5′-CCACGTAGTAGACGATGGGC-3′. The 18s rRNA was used for normalization, and the primers were as follows: sense 5′-ACACGGACAGGATTGACAGA-3′ and antisense 5′-GGACATCTAAGGGCATCACAG-3′. Real-time PCR was performed in a 96-well optical reaction plate using SYBR Premix Ex Taq™. Real-time PCR reactions were performed on Agilent Mx3000P QPCR Systems (Agilent, CA).

Fig. 2. SS-31 ameliorates glomerular pathology. A: kidney sections were stained with periodic acid Schiff or immunolabeled with anticollagen IV and fibronectin antibodies (original magnification: ×400). B: glomerular injury scores in diabetic mice. C: mean glomerular tuft volume. D: semiquantitative expression levels of collagen IV and fibronectin. E: expression level of fibronectin in kidney cortex tissues was analyzed by Western blot. All values are expressed as means ± SE. **P < 0.01 vs. N Group. #P < 0.05 vs. STZ Group.
Western blot analysis. Kidney cortex tissues and MMCs were washed twice with ice-cold PBS with 1 mmol/l Na3VO4 and then treated for 60 min with ice-cold lysis buffer (20 mmol/l Tris-HCl, pH 7.4, 2.5 mmol/l EDTA, 1% Triton X-100, 10% glycerol, 1% deoxycholate, 0.1% SDS, 10 mmol/l Na2P2O7, 50 mmol/l NaF, 1 mmol/l Na3VO4, and 1 mmol/l phenylmethanesulfonyl fluoride). The cell lysates were centrifuged at 14,000 g for 25 min at 4°C, and the supernatants were collected. The protein concentration was measured by Bradford’s method. The cell lysates (50 μg of protein/lane) were loaded, separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were incubated overnight at 4°C with primary antibodies for fibronectin or β-actin, Bax/β-actin, Bcl-2/β-actin, Bax (1:1,000 dilution), TRX2 (1:1,000 dilution), Nox4 (1:1,000 dilution), TGF-β1 (1:1,000 dilution), p-CREB (1:1,000 dilution), CREB (1:1,000 dilution), caspase-3 (1:1,000 dilution), p-p38 MAPK (1:1,000 dilution), p38 MAPK (1:1,000 dilution), TXNIP (1:1,000 dilution), cleaved caspase-3 (1:1,000 dilution), Bax (1:1,000 dilution), Bcl-2 (1:1,000 dilution), and anti-Bax and Bcl-2 antibodies, and then scanned using the Odyssey Fc System (LI-COR, Lincoln, NE). The intensity of the bands was measured using LabWorks 4.5 software (UVP, Upland, CA).

Cell viability assessment. Cell viability was assessed at 48 h after HG exposure by trypan blue exclusion assay, as previously described (8). The percentage of cell viability was calculated as the number of live cells divided by the total number of live and dead cells multiplied by 100. Cell viability values were expressed as a percentage of control.

Apoptosis assays. Confluent mesangial cell monolayers were trypsinized and centrifuged at 190 g for 10 min. Cell pellets were gently resuspended in ice-cold 70% ethanol/30% PBS. Fixed cells were centrifuged at 190 g for 5 min, washed once in PBS, and resuspended in 200 μl of propidium iodide (200 μg/ml) and RNase A (500 μg/ml). Cells were incubated at 37°C for 1 h. About 1×10⁶ cells were analyzed using a flow cytometer (BD Immunocytometry Systems, Franklin Lakes, NJ).

Apoptosis of MMCs was also evaluated with in situ apoptosis detection kit. Cells were cultured on 8-well Lab-Tek chamber slides (Nalge Nunc, Rochester, NY) under the different experimental conditions for 48 h. It was performed by using DeadEnd Fluorometric TUNEL System, according to the manufacturer’s instructions. For quantification of positive TUNEL signals, a minimum of 500 cells was counted per well (n = 6), and the percentage of positively labeled cells was calculated.

Fig. 3. Effects of SS-31 on apoptosis, Bcl-2 and Bax expression in diabetic kidneys. A: apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), and the expression Bcl-2 and Bax was examined by immunohistochemistry. B: mean apoptotic cells per field (×400). C: expression levels of Bax and Bcl-2 mRNA were analyzed by real-time PCR. D: Western blot analysis was done with anti-Bax and Bcl-2 antibodies, and pixel densities were normalized against β-actin. Values are expressed as means ± SE. **P < 0.01 vs. N Group. #P < 0.05 vs. STZ Group.
Determination of cyt c release from mitochondria. Cytosol fraction was prepared as described previously (22). MMCs were incubated in the buffer containing 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 20 μM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Following incubation for 10 min in an ice bath, the cells were homogenized with the homogenizer for 30 strokes, and a buffer (300 μl) containing 200 mM mannitol, 70 mM sucrose, 5 mM EDTA, 5 mM Tris-HCl, pH 7.5, was added to the homogenates. After centrifugation at 1,000 g for 10 min at 4°C, the supernatants were centrifuged at 15,000 g for 30 min. The supernatants were collected and used as the cytosol fraction. The Cyt c level in the cytosol fraction was detected by Western blot analysis.

Enzyme-linked immunosorbent assay. After the cells were cultured in six-well plates under the different experimental conditions for 48 h, the supernatants were collected. The TGF-β1 or fibronectin protein was quantified using a commercial Quantikine ELISA kit for TGF-β1 (R&D Systems, Minneapolis, MN) or competitive sandwich ELISA for fibronectin (Chemicon International, Temecula, CA), according to the manufacturer’s descriptions.

Intracellular ROS and ATP content assay. The intracellular formation of ROS was detected using the fluorescence probe 5(and 6)chloromethyl-2,7’-dichlorodihydrofluorescein diacetate (CM-DCHF-DA; Invitrogen, Carlsbad, CA). After they were cultured in 6-well plates under the different experimental conditions for 48 h, the cells were washed, trypsinized, suspended in PBS, loaded with 10 μM DCHF-DA, and incubated at 37°C for 30 min. The measurement of ROS was performed using a flow cytometer (BD Immunocytometry Systems). Cellular ATP content was determined using an ATP bioluminescent assay kit (Sigma), according to manufacturer’s instructions.

Measurement of ΔΨm by flow cytometry. To measure the ΔΨm of MMCs, we used a fluorescent probe (JC-1; Molecular Probes), as described previously (40). Briefly, MMCs were collected by trypsinization, washed in PBS, and incubated with 1.0 μg/ml JC-1 for 15 min at 37°C. MMCs were then washed in PBS and analyzed immediately by a flow cytometer (BD Immunocytometry Systems). Photomultiplier settings were adjusted to detect green fluorescence (λem = 525 nm) of JC-1 monomer on filter 1 and red fluorescence (λem = 590 nm) of JC-1 aggregates on filter 2. The ratio of red/green fluorescence intensity values was used to assess ΔΨm.

TRX activity. Thioredoxin activity was measured using the insulin disulfide reduction assay, as described previously (21). After the cells were cultured in six-well plates under the different experimental conditions for 48 h, total cellular protein was extracted with lysis buffer. Forty microliters of reaction mixture [200 μl of HEPES buffer (1 M, pH 7.6), 40 μl of EDTA, 20 μl PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Following incubation for 10 min in an ice bath, the cells were homogenized with the homogenizer for 30 strokes, and a buffer (300 μl) containing 200 mM mannitol, 70 mM sucrose, 5 mM EDTA, 5 mM Tris-HCl, pH 7.5, was added to the homogenates. After centrifugation at 1,000 g for 10 min at 4°C, the supernatants were centrifuged at 15,000 g for 30 min. The supernatants were collected and used as the cytosol fraction. The Cyt c level in the cytosol fraction was detected by Western blot analysis.

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NADPH oxidase activity assay. NADPH oxidase activity was measured using the lucigenin-enhanced chemiluminescence method, as previously described (12). Kidney cortex or MMC homogenates were prepared in 1 ml or 250 μl, respectively, of lysis buffer (20 mM KH₂PO₄, pH 7.0, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin) by using a Dounce homogenizer. Twenty-five microliters of homogenate were added to 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin, and 100 μM NADPH at a final volume of 1 ml. Photon emission was measured every 30 s for 5 min in a luminometer. Superoxide production was expressed in relative light units per milligrams of protein (RLU/mg).

Statistical analysis. All experiments were performed at least three times. Data are expressed as means ± SE. Statistical analysis was performed by one-way ANOVA. Statistical significance was defined as P < 0.05.

RESULTS

Effects of SS-31 on animal characteristics. There was a significant increase in blood glucose in all STZ-injected mice compared with nondiabetic (N) control mice. Treatment with SS-31 showed no effect on the elevated blood glucose level (Fig. 1A). Urinary albumin excretion (UAEx) was about 5.5-fold higher in STZ Group than that of the N Group, and treatment with SS-31 significantly alleviated diabetic mice UAEx (Fig. 1B). The kidney-to-body weight ratio was increased in STZ Group compared with that of the N Group; SS-31 treatment was associated with a significant reduction in the kidney-to-body weight ratio (Fig. 1C). Compared with the N Group, the urinary 8-OHdG level was increased in the STZ Group, and treatment with SS-31 significantly reduced 8-OHdG excretion (Fig. 1D).

Effects of SS-31 on changes of histopathology and expression of fibronectin and collagen IV. When compared with N Group mice, PAS-stained kidneys of STZ Group mice dis-
played prominent mesangial expansion and glomerular hypertrophy, which was ameliorated by SS-31 treatment (Fig. 2, A–C). Immunohistochemistry staining showed higher glomerular levels of both fibronectin and collagen IV in the STZ Group vs. the N Group, and SS-31 reduced the expression of fibronectin and collagen IV in diabetic kidneys (Fig. 2, A and D). Likewise, on the basis of Western blot analysis, fibronectin expression increased in kidney cortex in STZ Group compared with N Group, and this increase was partially attenuated by SS-31 treatment (Fig. 2E).

Effects of SS-31 on apoptosis, Bcl-2 and Bax expression in diabetic kidneys. An increased apoptosis was observed in diabetic kidney, as assessed by TUNEL assay. Apoptotic cells were mainly confined to cortical tubules and were rarely seen in renal glomeruli. No apparent apoptotic cells were seen in the N Group. The treatment of SS-31 significantly reduced the number of apoptotic cells in diabetic kidneys (Fig. 3, A and B). Furthermore, we observed the effect of SS-31 on expression of Bax and Bcl-2 protein and mRNA in diabetic kidney by immunohistochemistry, immunoblotting, and real-time PCR. Bax protein and mRNA expression in kidneys from the STZ Group was increased compared with N Group, and increased expression of Bax protein and mRNA was significantly inhibited by SS-31 treatment. Conversely, the Bcl-2 protein and mRNA expression was notably decreased in STZ Group compared with N Group; SS-31 treatment significantly promoted the expression of Bcl-2 protein and mRNA in diabetic kidneys (Fig. 3, A, C, and D).

Effect of SS-31 on expression of TGF-β1 and TXNIP in diabetic kidneys. Next, we evaluated the role of SS-31 on TGF-β1 and TXNIP expression in diabetic kidneys. As shown in Fig. 4A, diabetes was associated with an increase in renal cortical expression of TGF-β1 and TXNIP protein, and this increase was partially attenuated by treatment with SS-31. Furthermore, we also evaluated the effect of SS-31 on TGF-β1 and TXNIP mRNA expression by real-time PCR. The expression of TGF-β1 and TXNIP mRNA increased in diabetic renal cortex compared with N Group; the increased expression of TGF-β1 and TXNIP mRNA was diminished by SS-31 treatment (Fig. 4B).

Effects of SS-31 on activation of p38 MAPK and CREB, Nox4 expression and NADPH oxidase activity in diabetic kidneys. Phosphospecific p38 MAPK levels in renal cortex were significantly greater in the STZ Group compared with the modulation of p38 MAPK and CREB, Nox4 expression, and NADPH oxidase activity by SS-31 treatment. A: expression of p-p38 MAPK and p-CREB was detected by Western blot analysis, and the relative intensity of p-p38 MAPK and p-CREB was normalized to the total p38 MAPK and CREB, respectively. B: expression of Nox4 was detected by Western blot analysis, and the relative intensities were normalized against β-actin. C: NADPH oxidase activity was measured using a lucigenin-enhanced chemiluminescence assay. Values are expressed as means ± SE. **P < 0.01 vs. N Group. #P < 0.05 vs. STZ Group.
The increased expression of p-p38 MAPK was inhibited significantly by SS-31 treatment (Fig. 5A). To determine whether activation of the p38 MAPK pathway could induce parallel increases in the activation of a p38 MAPK target transcription factor, we examined the expression of CREB. As shown in Fig. 5A, the p-CREB expression was significantly higher in STZ Group relative to N Group. Similar to p38 MAPK, SS-31 treatment ameliorated the increase of p-CREB in diabetic renal cortices. Nox4 protein expression was increased in diabetic mice kidney cortex and was significantly prevented when mice were administered SS-31 (Fig. 5B). NADPH oxidase activity was also measured using a lucigenin-enhanced chemiluminescence assay. There was a significant increase in NADPH oxidase activity in diabetic kidney compared with N Group mice. Treatment with SS-31 significantly suppressed the increase of NADPH oxidase activity in diabetic renal cortices (Fig. 5C).

Effect of SS-31 on HG-induced cell injuries in MMCs. We examined the effect of SS-31 on HG-induced loss of cell viability using trypan blue exclusion assay. After treatment with 30 mM glucose for 48 h, there was a significant increase in cell death (Fig. 6A). The detrimental effects of HG on cell viability were abolished by SS-31 treatment. Mannitol had no effect on cell viability. Next, we evaluated the effect of SS-31 on

**Fig. 6. SS-31 inhibits HG-induced cell injuries in MMCs. MMCs were incubated with normal glucose (NG; 5.6 mM), NG+24.4 mM mannitol (M), M+SS-31 (100 nM, M+SS-31), high glucose (HG; 30 mM), and HG+SS-31 (100 nM, HG+SS-31) for 48 h. A: cell viability was examined by trypan blue exclusion assay. HG treatment causes loss of cell viability in MMCs, and SS-31 prevented the increased cell death under HG condition. B: apoptosis was detected by flow cytometry. C: quantitative analysis of the effect of SS-31 on HG-induced apoptosis by TUNEL assay. SS-31 significantly decreased the HG-induced cell apoptosis. D: expression of cleaved caspase-3, caspase-3, Bcl-2, and Bax was analyzed by Western blot. The relative intensity of cleaved caspase-3 was normalized to the caspase-3. Normalization for Bax and Bcl-2 against β-actin was made before calculating the Bax/Bcl-2 ratio. SS-31 significantly reduced HG-induced expression of cleaved caspase-3 and Bax/Bcl-2 ratio. E: Western blot analysis of cytochrome c (Cyt c) in cytosolic fractions of MMCs. The relative intensity of Cyt c was normalized to the β-actin. Cyt c in the cytosol fraction was increased when MMCs were exposed to HG at 48 h and this increase was markedly attenuated by treatment with SS-31. Values are expressed as means ± SE (n = 6). **P < 0.01 vs. NG; #P < 0.05 vs. HG.**
HG-induced apoptosis in MMCs under HG condition by flow cytometry and TUNEL assay. Compared with the control group, HG significantly promoted cell apoptosis, and this increase was markedly attenuated by treatment with SS-31 in MMCs (Fig. 6, B and C). We further examined whether HG-induced cell apoptosis was linked to expression of cleaved caspase-3, Bax, and Bcl-2. Compared with those of the NG Group, the expression of cleaved caspase-3 and ratio of Bax/Bcl-2 significantly increased in the HG Group; whereas treatment with SS-31 significantly suppressed HG-induced expression of cleaved caspase-3 and ratio of Bax/Bcl-2 in MMCs (Fig. 6D). Western blot analysis of subcellular extracts showed that Cyt c in the cytosol fraction was increased when MMCs were exposed to HG at 48 h, and this increase was markedly attenuated by treatment with SS-31 (Fig. 6E). The result indicated that HG induced the release of Cyt c from mitochondria to the cytoplasm, and SS-31 effectively reduced the release of Cyt c from mitochondria to cytoplasm induced by HG. In addition, mannitol had no effect on cell apoptosis, expression of cleaved caspase-3, ratio of Bax/Bcl-2, and release of Cyt c. Taken together, these results suggest that SS-31 can suppress the mitochondrial pathway of apoptosis during HG treatment to protect MMCs.

Effect of SS-31 on HG-induced synthesis of TGF-β1 and fibronectin in MMCs. TGF-β1 and fibronectin levels in the culture medium were measured with ELISA. A significant increase in TGF-β1 and fibronectin protein was seen after MMCs were cultured under HG condition at 48 h, whereas the HG-induced overexpression of TGF-β1 and fibronectin was significantly inhibited by SS-31 treatment (Fig. 7, A and B). Meanwhile, MMCs in HG Group showed a significant higher mRNA level of TGF-β1 than that in the NG Group, and SS-31 significantly decreased the HG-induced overexpression of TGF-β1 mRNA in the MMCs (Fig. 7C).

Effects of SS-31 on HG-induced ΔΨm and ATP alterations and ROS production. As shown in Fig. 8A, MMCs incubated with HG showed a time-dependent change of ΔΨm in mesangial cells stimulated with high glucose (30 mM). ΔΨm levels increased at 6 h after HG stimulation, then decreased at 12 h, and remained at low levels until 24–48 h. Treatment with SS-31 significantly reversed HG-induced alteration of ΔΨm in MMCs at 6 h, 24 h, and 48 h. The changes of cellular ATP content are similar with ΔΨm. SS-31 treatment also could reverse HG-induced alteration of ATP levels at 6 h, 24 h, and 48 h (Fig. 8B). Next, we evaluated the effect of SS-31 on ROS production in MMCs under HG condition. As shown in Fig. 8C, the intracellular ROS production in MMCs increased significantly after stimulation with HG for 48 h. In contrast, ROS production was remarkably suppressed with SS-31 treatment. These data suggested that SS-31 prevented ROS generation, ΔΨm loss, and ATP alteration in MMCs caused by an HG environment.

Effects of SS-31 on HG-induced expression of TXNIP and TRX2, and TRX activity in MMCs. Our previous study has demonstrated that TXNIP was involved in HG-induced MMC apoptosis, and knockdown of TXNIP reversed HG-induced reduction of TRX activity (44). To examine whether TXNIP contributed to the protective effect of SS-31 on MMCs under HG, real-time PCR and Western blot were used to measure the expression of TXNIP. After treated with HG for 48 h, the expression of TXNIP mRNA and protein increased significantly, whereas treatment with SS-31 significantly suppressed HG-induced expression of TXNIP in MMCs (Fig. 8, D and E).

Next, we observed the effect of SS-31 on the expression of TRX2 in MMCs exposed to HG. As shown in Fig. 8E, HG had no effect on expression of TRX2. However, treatment with SS-31 promoted the expression of TRX2 in MMCs under the HG condition. Furthermore, we evaluated the effect of SS-31 on activity of TRX in MMCs under HG. As shown in Fig. 8F, after 48 h of exposure to HG, the biologic activity of TRX was significantly reduced; whereas SS-31 treatment could reverse the reduction of TRX biologic activity.

Effect of SS-31 on HG-induced activation of p38 MAPK and CREB in MMCs. To investigate the modulation of activation of p38 MAPK and CREB by SS-31 under HG condition, MMCs were cultured with HG and treated with SS-31 for 48 h. Compared with the NG Group, the activation of p38 MAPK and CREB significantly increased in HG Group, whereas SS-31 treatment significantly suppressed HG-induced phosphorylation of p38 MAPK and CREB (Fig. 9A). Next, we observed the effect of p38 MAPK inhibition on activation of CREB in MMCs exposed to HG. As shown in Fig. 9A, the HG-induced phosphorylation of CREB was ameliorated significantly by p38 MAPK inhibitor SB203580 treatment.

Effect of SS-31 on HG-induced Nox4 expression and NADPH oxidase activity in MMCs. Next, we evaluated the effect of SS-31 on HG-induced Nox4 expression and
After treatment with 30 mM glucose for 24 h, there was a significant increase in Nox4 expression and NADPH oxidase activity (Fig. 9, B and C). The HG-induced increase of Nox4 expression and NADPH oxidase activity was significantly reduced by treatment with SS-31 in MMCs (Fig. 9, B and C).

NADPH oxidase activity. After treatment with 30 mM glucose for 24 h, there was a significant increase in Nox4 expression and NADPH oxidase activity (Fig. 9, B and C). The HG-induced increase of Nox4 expression and NADPH oxidase activity was significantly reduced by treatment with SS-31 in MMCs (Fig. 9, B and C).

DISCUSSION

In the present study, we demonstrate that administration of mitochondria-targeted antioxidant peptide SS-31 to diabetic mice alleviated renal hypertrophy, renal cell apoptosis, and subsequently suppressed superoxide production, indicating
beneficial effects of SS-31 on diabetic nephropathy. Further, SS-31 inhibits HG-induced ROS generation, ATP alteration, Cyt c release from mitochondria to cytoplasm, expression of TXNIP, Nox4 and activation of p38 MAPK and CREB, and NADPH oxidase activity, and upregulates expression of TRX2, stabilizes TRX biologic activity and protects against mesangial cell apoptosis in response to hyperglycemia.

Increased ROS production is a major cause of microvascular and macrovascular complications leading to disability and death in patients with Type 2 diabetes (6, 50). Accumulation of ROS plays a critical role in the pathogenesis of diabetic nephropathy (17). SS-31, a small mitochondria-targeted peptide, can be selectively concentrated in the inner mitochondrial membrane and reduce mitochondrial ROS generation (46, 54, 55). In a rat model of ischemia-reperfusion injury, treatment with SS-31 accelerated recovery of ATP, reduced apoptosis and necrosis of tubular cells, decreased oxidative stress and the inflammatory response, and abrogated tubular dysfunction (47). Glomerular hypertrophy has been well characterized in the early STZ-induced diabetes and is closely linked to glomerular hyperfiltration and microalbuminuria in diabetic mice (34, 51). In the present study, our results demonstrated that STZ-induced diabetic mice exhibited significantly increased UAE, kidney-to-body weight ratio, and mean glomerular volume at 8 wk after the onset of diabetes. Administration of SS-31 to diabetic mice alleviated albuminuria, renal hypertrophy, and urinary 8-OHdG level. We also found that SS-31 administration diminished mesangial expansion and expression of fibronectin and collagen IV in diabetic mice, indicating beneficial effects of SS-31 on the diabetic kidney.

Apoptosis contributes to the development of diabetic nephropathy (41). Previous evidence suggests that HG-induced cellular apoptosis and mesangial depletion was a major mechanism that led to renal injury in diabetics (26, 31). HG induces ROS generation, and ROS contribute to mesangial cell apoptosis (26). One of the main contributors of apoptosis is the increase in ROS, which increases intracellular DNA damage and, ultimately, can result in the onset of apoptosis. SS-31 has been shown to prevent apoptosis that is induced by a lipid hydroperoxide in neuronal cell lines (54). SS-31, which is currently in clinical trials for ischemia-reperfusion injury, protects mitochondrial cristae by interacting with cardiolipin on
CREB and ATF-2 (2). CREB is a member of transcription factor family, which converts rapid and transient signals into long-term changes in gene expression by binding to the cAMP response element. Previous studies showed that phosphorylation of the transcription factor CREB was induced in diabetic kidneys and mesangial cells (16, 25). In our study, similar to p38 MAPK activity, SS-31 treatment ameliorated the increase of CREB activity in diabetic kidneys and mesangial cells exposed to HG. Taken together, these results suggest that SS-31 attenuated diabetes-induced activation of p38 MAPK and its downstream target transcription factor CREB, which has been implicated in the pathogenesis of DN.

However, a few limitations of the present study should be addressed: 1) inflammation plays an important role in diabetic nephropathy. We did not examine the effect of SS-31 on inflammation in the diabetic kidney; 2) the expression of protein and mRNA and NADPH oxidase activity are performed on kidney cortex tissue, so we cannot define which cells are affected by the diabetes or treatment; 3) blood pressure in mice was not measured; 4) in the in vitro experiment, very high glucose concentrations (30 mM) are used (if 20–25 mM glucose concentrations had been used, the results would have been strengthened.); and 5) in this study, we found SS-31 could inhibit the expression of TXNIP in diabetic kidney or mesangial cell under the HG condition; however, the data are suggestive and only show an association and not cause and effect.

In summary, our data demonstrate that SS-31 alleviated renal hypertrophy, reduced UAE, slowed the accumulation of extracellular matrix protein fibronectin and collagen IV, and inhibited renal cell apoptosis in diabetic mice. The in vitro experiment revealed that SS-31 normalized HG-induced enhanced ROS production, cell apoptosis, and alteration of ΔΨm and ATP in MMCs. SS-31 also inhibited expression of TXNIP and Nox4, activation of p38 MAPK, and CREB and NADPH oxidase activity in diabetic kidney and mesangial cells exposed to HG. So, we can infer that SS-31 may have therapeutic benefits for diabetic nephropathy.

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DISCLOSURES

No conflict of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: Y.H., S.L., M.W., J.W., Y.R., H.W., and C.H. performed experiments; Y.H. and Y.S. interpreted results of experiments; Y.H. drafted manuscript; S.L., C.D., C.H., H.D., and Y.S. prepared figures; Y.S. conception and design of research; C.D., H.D., and Y.S. analyzed data; C.D., H.D., and Y.S. interpreted results of experiments; C.D., H.D., and Y.S. edited and revised manuscript; Y.S. approved final version of manuscript.

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