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Mitochondria-targeted antioxidant peptide SS31 attenuates high glucose-induced injury on human retinal endothelial cells

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ABSTRACT

Purpose: To investigate the effect of mitochondria-targeted antioxidant peptide SS31 on prevention of high glucose-induced injury on human retinal endothelial cells (HRECs).
Methods: Cultured P3–P5 HRECs were divided into three groups: 5 mM glucose group, 30 mM glucose group and 30 mM glucose co-treated with 100 nM SS31 group. 24 and 48 h after treatment, Annexin V-FITC/PI staining was used to evaluate the survival of HRECs. Overproduction of ROS was assessed by MitoSOX staining under confocal microscope. Change of mitochondrial potential ($\Delta\Psi_m$) of HRECs was measured by flow cytometry after JC-1 fluorescent probe staining. Release of cytochrome c was assessed by confocal microscopy and western blot. Expression of caspase-3 and thioredoxin-2 (Trx-2) were measured by western blot and real-time PCR.
Results: Compared to the high glucose group, co-treatment with 100 nM SS31 significantly protected HRECs from high glucose-induced injury, reduced the production of ROS in mitochondria, stabilized $\Delta\Psi_m$, decreased the release of cytochrome c from mitochondria to cytoplasm, decreased the expression of caspase-3 and increased the expression of Trx-2 in high glucose-treated HRECs.
Conclusions: SS31 attenuates the high glucose-induced injuries on HRECs by stabilizing $\Delta\Psi_m$, decreasing ROS production, preventing the release of cytochrome c from mitochondria, decreasing the expression of caspase-3 and increasing the expression of Trx-2. Our study suggests that SS31 may be as a potential new treatment for diabetic retinopathy and other oxidative stress-related diseases.

1. Introduction

Diabetic retinopathy (DR) is one of the most prominent microvascular complications for patients with type 1 or type 2 diabetes and a leading cause of visual impairment throughout the world [1,2]. Until now, there is no effective therapy for the vision loss associated with DR.

Recent studies have suggested that hyperglycemia results in increased production of reactive oxygen species (ROS). ROS are important to the development of diabetes and diabetic complications such as retinopathy and nephropathy [3]. ROS are normally kept under control by endogenous antioxidant systems including ascorbic acid, glutathione, superoxide dismutase (SOD) and catalase. Oxidative stress occurs when the balance between ROS and antioxidants is perturbed [4]. Excessive ROS can damage cells by oxidizing membrane phospholipids, proteins and nucleic acids which trigger apoptotic pathways and subsequently lead to cell death. Mitochondria are essential organelles and regulate a number of key processes including calcium homeostasis and redox control. Mitochondria are both the major endogenous source of intracellular ROS and the primary target of oxidative damage [5]. Our previous study also demonstrated that HRECs produce excessive endogenous ROS resulting in further mitochondrial DNA damage and additional ROS production when exposed high glucose [6]. So these studies suggested that protecting mitochondria from oxidative injury may provide a promising therapeutic strategy to develop effective treatments for DR. Recently, several antioxidants have been attempted to treat DR. However, most of these efforts have not achieved significant clinical benefits. The explanation for these unsatisfactory outcomes may reflect the failure of traditional antioxidants to adequately target and penetrate mitochondria.

To effectively protect mitochondrial functions and prevent mitochondrial oxidative stress, drug therapy needs to target to mitochondria. SS31 peptide (H-D-Arg-Dmt-Lys-Phe-NH_2), accidentally discovered in studies on opioid receptor targeted peptides by Hazel H. Szeto and Peter W. Schiller, is a novel class of mitochondria-targeted

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antioxidant peptide. The structural motif of SS31 centers on alternating aromatic residues and basic amino acids. SS31 can scavenge H_2O_2 and ONOO– and inhibit lipid peroxidation [7].

In this study, we aim to investigate if SS31 is able to attenuate high glucose-induced HRECs injuries. To our knowledge, this is the first time such a study is being reported.

2. Materials and methods

2.1. HRECs culture and treatment

Cultured HRECs were obtained from human donors' eyes in the Eye Bank of Zhongshan Ophthalmic Center. The procedures of harvesting HRECs were described previously [8]. Cells resuspended in human endothelial serum free medium (HE-SFM, Gibco, NY, USA) with 10% fetal bovine serum (FBS), 5 ng/ml recombinant human β-endothelial cell growth factor (β-ECGF) (R&D Systems Inc., MN, USA) and 1% insulin-transferrin-selenium (Gibco, NY, USA) were plated into a fibronectin-coated flask and incubated at a 37 °C with humidified atmosphere containing 5% CO_2. Cultured cells underwent FITC-vWF (a specific marker of endothelial cells) immunostaining for assessing the cell purity by flow cytometry. The percent of vWF positive staining cells is about (92.8 ± 4.8%).

The HRECs were maintained with HE-SFM supplemented with 10% FBS and 5 ng/ml β-ECGF. The HRECs were divided into three groups: 5 mM glucose (normal glucose) control group, 30 mM glucose (high glucose) group, 30 mM glucose co-treated with SS31 (100 nM). SS31 was provided by Stealth Peptides International, Shanghai, PR China. After reached 70% confluence, passage 3–5 HRECs were incubated with different culture medium for 24 and 48 h for the experiments.

2.2. Survival of HRECs by flow cytometry

To find the optimum concentration of SS31 that was able to protect HRECs from high glucose-induced cell death, the 30 mM glucose group was co-treated with four different concentrations of SS31 (10 nM, 100 nM, 1 μM, or 10 μM). 24 h after treatment, survival ratio of HRECs was determined by Annexin V and PI staining according to the manufacturer's instructions (Bender Medsystems, Vienna, Austria). HRECs were suspended in 200 μl of 1× binding buffer and then added 5 μl of annexin V and 10 μl of PI, vortexed and incubated for 15 min. Finally, 200 μl of 1× binding buffer was added and samples were evaluated by flow cytometry.

2.3. Measurement of $\Delta\Psi_m$ by flow cytometry

JC-1 fluorescent probe (Molecular Probes, OR, USA) was used to measure the $\Delta\Psi_m$ of HRECs. HRECs were incubated with 1.0 μg/ml JC-1 for 15 min at 37 °C and then pelleted at 800 g for 5 min, washed with PBS and analyzed immediately by BD FACS Aria™ flow cytometer. Photomultiplier settings were adjusted to detect green fluorescence (Uem = 525 nm) of JC-1 monomers on filter 1 and red fluorescence (Uem = 590 nm) of JC-1 aggregates on filter 2. The ratio of red:green (aggregate:monomer) fluorescence intensity values was used to assess $\Delta\Psi_m$.

2.4. Measurement of ROS level in live HRECs

MitoSOX™ red reagent, which is oxidized by superoxide and exhibits red fluorescence in the mitochondria, was used to localize the intracellular ROS. A 5 mM MitoSOX™ reagent stock solution was prepared by adding 13 μl of dimethylsulfoxide (DMSO) to one vial of MitoSOX™ mitochondrial superoxide indicator (50 μg). Next, 1.0 ml of 5 μM MitoSOX™ reagent working solution was applied to live HRECs which were adhered to cover slips and incubated for 10 min at 37 °C. Finally, the cells were gently washed with warm buffer and viewed under a Zeiss LSM510 confocal microscope.

2.5. Detection of cytochrome c release by confocal immunofluorescence

HRECs were fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, blocked with goat serum for 1 h and then incubated at 4 °C overnight with mouse monoclonal anti-cytochrome c and rabbit polyclonal anti-HSP60 (a mitochondria-specific protein) (Santa Cruz Biotechnology Inc., CA, USA) antibodies. Finally, the cells were incubated with cy2-conjugated anti-mouse and cy3-conjugated anti-rabbit secondary antibodies (BD Biosciences, CA, USA) for 45 min at RT. Cells were then washed, mounted and photographed under a confocal microscope.

2.6. Measurement of cytochrome c release and protein expression of Trx-2 and caspase-3 by western blot

Protein samples for detecting the cytochrome c release: subcellular fractionation was used to detect cytochrome c content in cytosol and mitochondria by Western blotting [9]. Briefly, HRECs were suspended with Buffer A [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM Sucrose and 1× protease inhibitor cocktail (Sigma–Aldrich, MO, USA)] and homogenized using a Dounce homogenizer. Unbroken cells and nuclei were removed by centrifugation at 1000*g* for 10 min at 4 °C. The supernatant was further centrifuged at 10,000*g* for 20 min. The supernatant of 10,000*g* spin was removed to clean tubes and centrifuged at 100,000*g* for 1 h at 4 °C (the supernatant of this spin collected as cytosolic fraction). The 10,000 mitochondrial pellet was resuspended in Buffer A containing 0.5% (v/v) NP40 and collected as the mitochondrial fraction. The collected cytosolic and mitochondrial fractions were mixed with 2× sample loading buffer to prepare samples.

Protein samples for detecting protein expression of caspase-3 and Trx-2: Harvested HRECs were homogenized in 100 μL of lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100 and 1× protease inhibitor cocktail (Sigma–Aldrich, MO, USA) and then centrifuged at 11,000*g* at 4 °C for 30 min. Supernatants were collected and mixed with 2× sample loading buffer to prepare samples.

The protein samples were separated by sodium-dodecyl sulfate PAGE and subsequently transferred to polyvinylidene difluoride membrane (Bio-Rad, CA, USA). Membranes were blocked with 5% skim milk for 1 h at RT and incubated at 4 °C overnight with mouse monoclonal anti-cytochrome c (Cell Sciences, Canton, MA), rabbit polyclonal anti-Thioredoxin-2 (Trx-2) (provided by Dr. Wang Min, Yale University, New Haven, CT) and caspase-3 (Cell Signaling Technology Inc., MA, USA) antibodies. The membranes were then incubated with HRP-conjugated secondary antibody for 1 h at RT. The integrated optical density (OPTDI) of each detected protein band was normalized to those of internal control β-actin band for quantitative comparison.

2.7. mRNA expression of Trx-2 by Quantitative Real Time PCR

Total RNA was isolated from HRECs using Trizol reagent (Gibco, CA, USA) according to the manufacturer's instructions. RNA was treated with DNase I (Sigma–Aldrich, MO, USA) to remove any contaminating genomic DNA and transcripted to cDNA using reverse transcriptase (Takara, Siga, Japan). Real-time PCR was performed in a 96-well optical reaction plate (Bio-Rad, CA, USA) using SYBR Green qPCR SuperMix (Invitrogen, CA, USA). Real-time PCR reactions

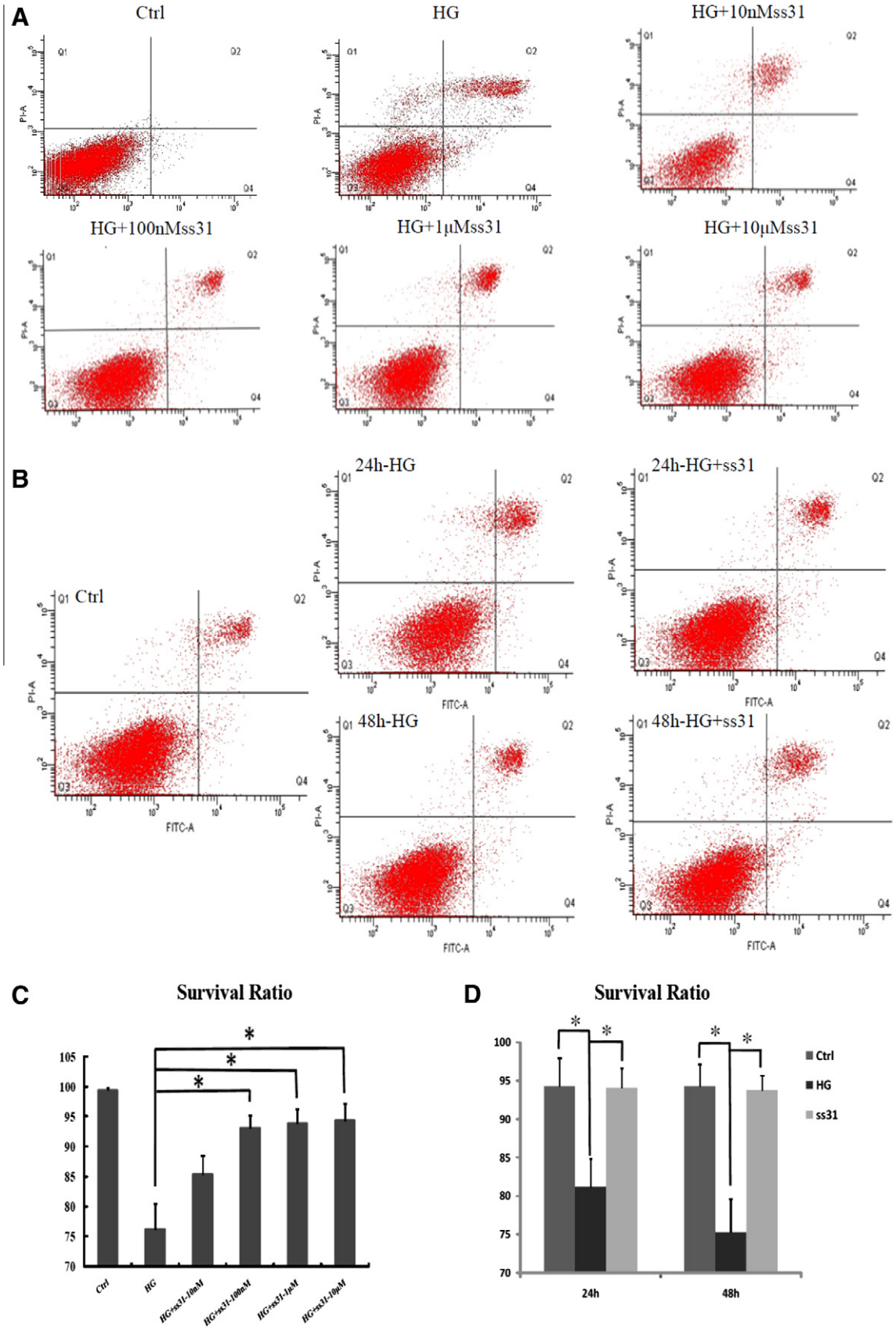

Fig. 1. SS31 protected HRECs from high glucose-induced cell death. (A) survival of HRECs in 30 mM high glucose (HG) co-treated with different concentrations of SS31 (10nM, 100 nM, 1 μM and 10 μM) for 24 h. Annexin V/PI staining analysis for apoptosis, showed that the survival ratios for HRECs (Q3) were (99.3 ± 0.5%), (76.2 ± 4.3%), (87.3 ± 3.2%), (93.1 ± 2.1%), (93.8 ± 2.5%) and (94.3 ± 2.8%), respectively 24 h after treatment. (C) Graphic representation of the survival ratios for HRECs. $^{*}p < 0.05$ vs. HG group. (B) when HRECs were exposed to 30 mM glucose for 24 and 48 h, the survival ratio of HRECs was increased markedly under the protection of 100 nM SS31. (D) Quantitative analysis of the survival ratio of HRECs at 24 and 48 h after treatment. $^{*}p < 0.05$ vs. HG group.

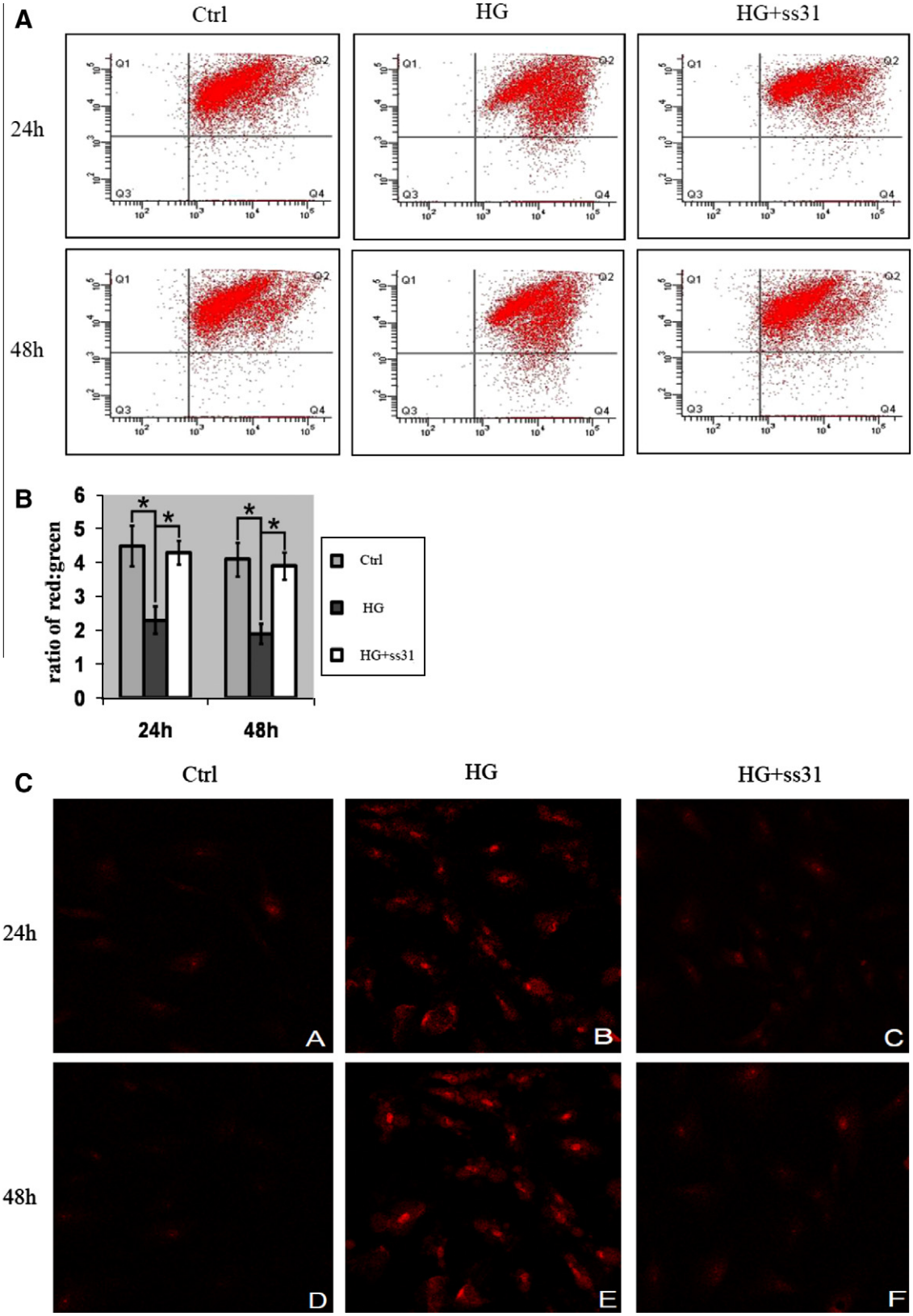

Fig. 2. SS31 prevented the mitochondrial potential loss and decreased ROS production of HRECs induced by high glucose. (A) The $\Delta\Psi_m$ of HRECs was measured by flow cytometry after JC-1 staining. High glucose treatment resulted in a rapid loss of $\Delta\Psi_m$ in HRECs at 24 and 48 h. In contrast, SS31 increased $\Delta\Psi_m$ compared with the HG group. (B) Quantitative analysis of $\Delta\Psi_m$ in HRECs for 24 and 48 h. Values represent mean ± SD of six separate experiments performed in triplicate. $^*p < 0.05$ vs. HG group. (C) Confocal microscopy was used to localize ROS production in mitochondria of HRECs. The ROS level of mitochondria in the normal glucose group was very low at 24 and 48 h (A, D). Exposure to 30 mM glucose for 24 and 48 h significantly increased production of ROS (B, E). 100 nM SS31 decreased the high glucose-induced mitochondrial ROS production (C, F).

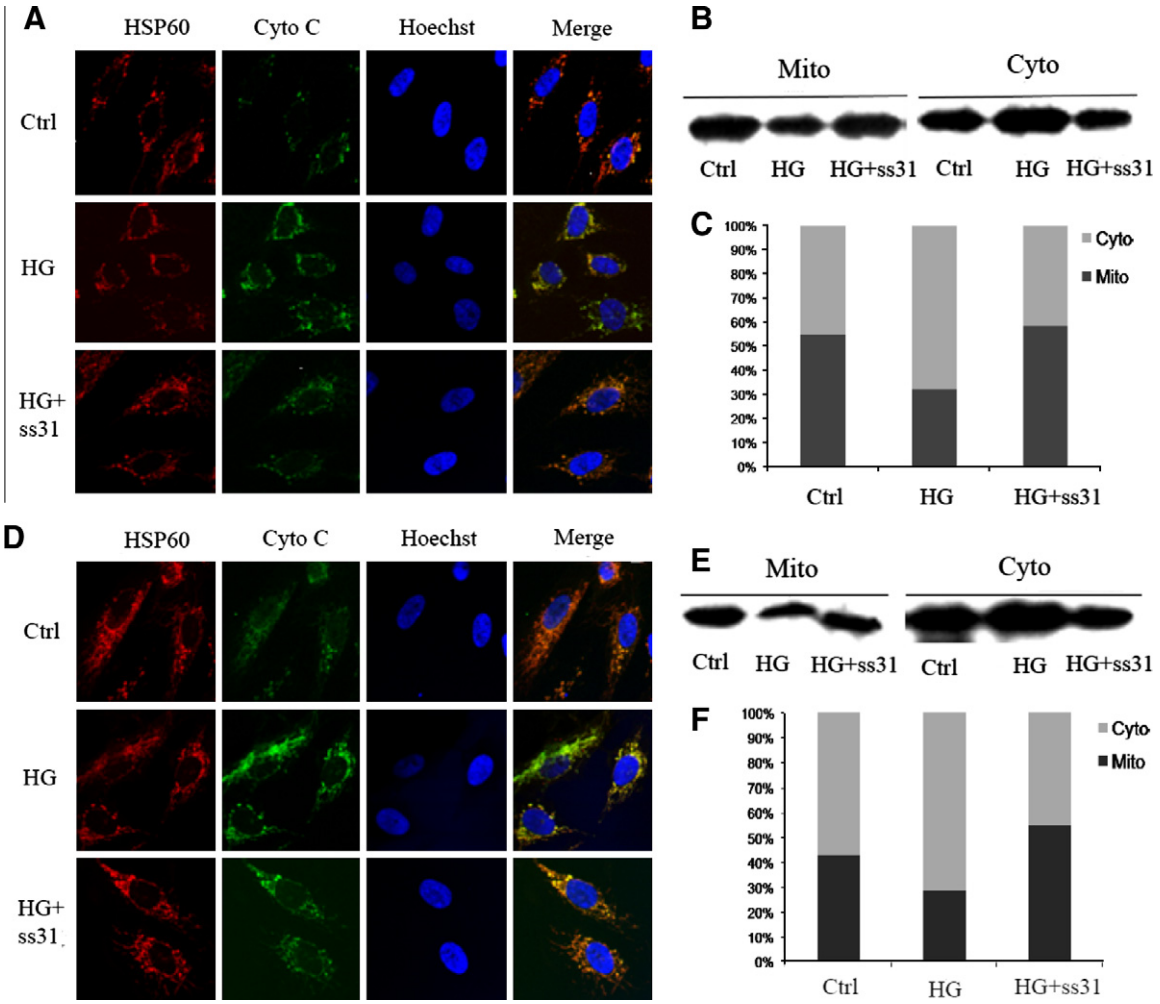

Fig. 3. SS31 reduced the release of cytochrome c from mitochondria to cytoplasm. (A, D) Confocal microscopic analysis showed that HRECs in the normal glucose group and the HG co-treated with SS31 group have high levels of overlapping cytochrome c staining (*green*) and HSP60 staining (*red*) at 24 and 48 h. When exposed to 30 mM glucose alone, cytochrome c was increased in the cytoplasm at 24 and 48 h. (B, E) Cytochrome c in mitochondria and cytoplasm was determined by Western blot. When exposed to high glucose for 24 and 48 h, sub-cellular extracts also indicated that more cytochrome c was released from mitochondria to cytoplasm. 100 nM SS31 decreased this translocation of cytochrome c from mitochondria to cytoplasm. (C, F) Quantitative analysis of the percentage of cytochrome c in mitochondria and cytoplasm of HRECs at 24 and 48 h.

were performed on ABI Prism 7000 sequence detection system (Applied Biosystems, CA). Data from multiple samples were normalized to ribosomal RNA (18S). Primers of Trx-2 were: F: 5′ ATCCTGACGGAAGACCAAG 3′ R: 5′ GCAATGAGGGTTGTGATGTG 3′.

2.8. Statistical analysis

All data are presented as mean ± SD. Normally distributed data were compared by independent two samples *t*-test or one-way ANOVA where appropriate. When a significant difference was detected between groups, multiple comparisons of means were performed using the Bonferroni procedure. Statistical analyses were performed using SPSS 15.0 statistics software. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Survival of HRECs in high glucose co-treated with SS31

Compared to the normal control group, exposure to high glucose resulted in a significant lower survival ratio. There was no difference between the high glucose and the high glucose plus 10 nM SS31 group. In contrast, there were greater HRECs survival ratios ($p < 0.05$) in the groups co-treated with 100 nM, 1 μM, and 10 μM of SS31. There was no significant difference between 100 nM and 1 μM and 10 μM SS31 (Fig. 1A, C). Thus, 100 nM SS31 was the lowest concentration to significantly protect against high glucose-induced cell death. Hence, only the 100 nM SS31 co-treated group was used for the following experiments.

When exposed to 30 mM glucose for 24 or 48 h, the percentage of surviving HRECs (Q3) was (81.2 ± 3.6%) and (75.3 ± 4.1%), respectively. In contrast, the percentage of surviving HRECs in the 100 nM SS31 co-treated group increased to (94.1 ± 2.6%) and (93.7 ± 1.9%) (Fig. 1B). The differences between the high glucose and SS31 co-treated groups at both time points were significant ($p < 0.05$, Fig. 1D).

3.2. SS31 prevented the mitochondrial potential loss and decreased ROS production in high glucose-treated HRECs

To determine if mitochondria are involved in protective effect of SS31 against high glucose-induced cell death, $\Delta\Psi_m$ was measured. After treated with high glucose for 24 or 48 h, a rapid loss of $\Delta\Psi_m$ in HRECs was detected by JC-1 fluorescent probe. In contrast, $\Delta\Psi_m$

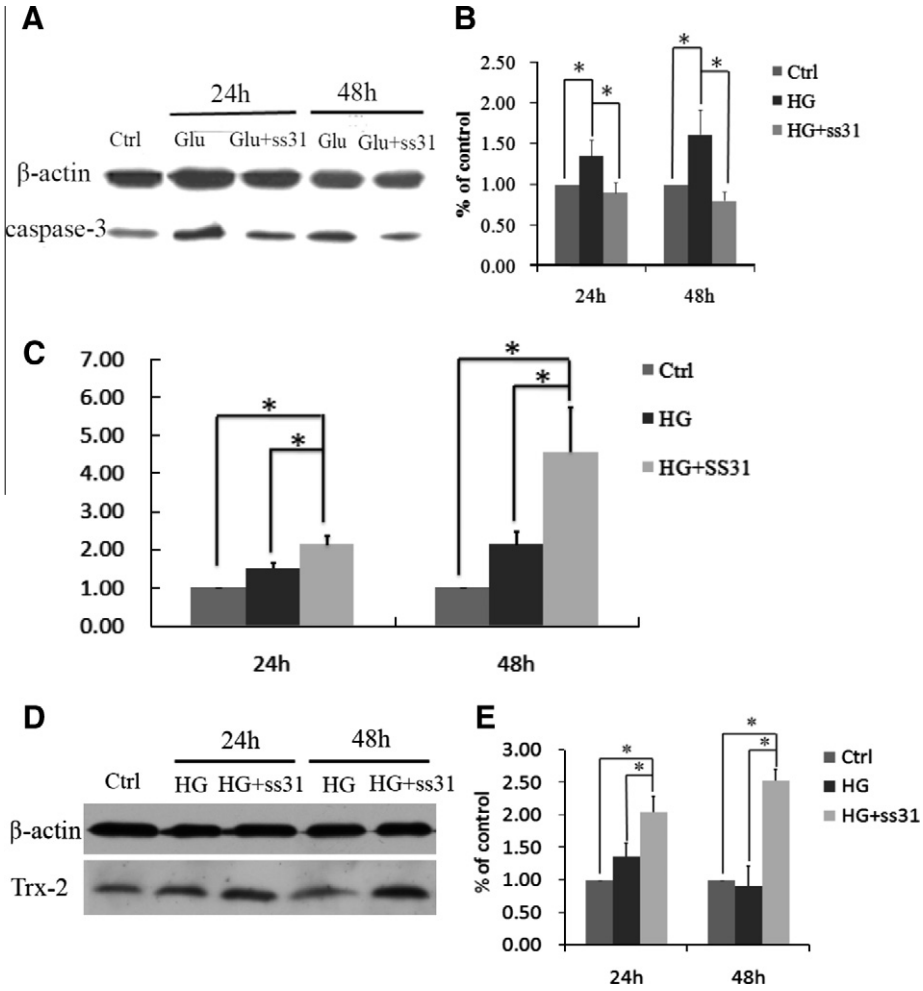

Fig. 4. The decreased expression of caspase-3 and increased expression of Trx-2 in HRECs under high glucose co-treated with SS31. (A) The protein expression level of caspase-3 was measured by Western blot. When HRECs were exposed to 30 mM glucose for 24 and 48 h, the expression level of caspase-3 increased dramatically. In contrast, the SS31 co-treated group showed significantly decrease in caspase-3 protein level compared to the HG group. (B) Quantitative analysis of the level of caspase-3 expression of HRECs. $^{*}p < 0.05$ vs. HG group. (C) The mRNA expression level of Trx-2 in HRECs was measured by quantitative real-time PCR. $^{*}p < 0.05$ vs. the normal glucose group and the HG group. Three independent samples were used for each time point. (D) The protein expression level of Trx-2 was measured by Western blot. The protein expression of Trx-2 in the HG co-treated with SS31 group significantly increased comparing to the normal glucose group. (E) Quantitative analysis of the protein level of Trx-2 in HRECs. $^{*}p < 0.05$ vs. the normal glucose group and the HG group.

of HRECs in the 100 nM SS31 co-treated group remained virtually unchanged (Fig. 2A, B). These data suggested that SS31 prevented $\Delta\Psi_m$ loss in HRECs caused by high glucose environment.

When treated with 30 mM glucose for 24 or 48 h, ROS production in mitochondria of HRECs increased significantly. In contrast, ROS production was remarkably reduced in the 100 nM SS31 co-treated group at both times (Fig. 2C).

3.3. SS31 inhibited the release of cytochrome c from mitochondria to cytoplasm

More cytochrome c (green) were observed in the cytoplasm of HRECs at 24 and 48 h after treatment with 30 mM glucose under confocal microscopy. This indicated that high glucose induces the release of cytochrome c from mitochondria to cytoplasm. In contrast, the overlapping of cytochrome c and HSP60 staining (yellow) was more prominent in HRECs of the normal and the high glucose SS31 co-treated group (Fig. 3A, D). This indicated that cytochrome c maintained its localization within the mitochondria. Thus, SS31 effectively reduced the release of cytochrome c from mitochondria to cytoplasm induced by high glucose. Western blot analysis of sub-cellular extracts also showed that more cytochrome c was released from mitochondria to cytoplasm when HRECs were exposed to high glucose at both 24 and 48 h (Fig. 3B, E) and SS31 inhibited the release of cytochrome c from mitochondria to cytoplasm.

3.4. SS31 decreased the expression of caspase-3 and increased the expression of Trx-2 in high glucose-treated HRECs

As seen in Fig. 4A, exposure to high glucose increased the expression of caspase-3 in HRECs whereas co-treatment with SS31 effectively inhibited the up-regulated expression of caspase-3 induced by high glucose.

To examine whether Trx-2 contributed to the protective effect of SS31 on HRECs under high glucose, real-time PCR and western blot were used to measure the expression of Trx-2. After treated with 30 mM glucose for 24 and 48 h, expression of Trx-2 mRNA was slightly but not significantly increased (Fig. 4C). However, this marginal increase may not be sufficient to normalize the over production of ROS. In contrast, the mRNA expression of Trx-2 in the high glucose co-treated with SS31 group increased 2.13- and 4.58-fold compared to the normal glucose group ($p < 0.05$). As shown in Fig. 4D, E, the protein expression of Trx-2 in the high glucose group co-treated with SS31 increased 2.05- and 2.53-fold compared to the

normal glucose group ($p < 0.05$). These data suggested that Trx-2 may be involved in the antioxidant effect of SS31 on high glucose-injured HRECs. This marked increase of Trx-2 in the SS31 co-treated group would help explain the reduction of mitochondrial ROS production when HRECs were exposed to SS31.

4. Discussion

Oxidative stress has been implicated in many diseases including ischemia–reperfusion injuries and diabetes [3,10,11]. It is considered to be one of the crucial contributors to the pathogenesis of DR [2]. Recently, the association between mitochondrial dysfunction and mitochondria-generated ROS has been described as part of the pathogenesis of DR [6,12,13]. So antioxidants have been proposed as treatment for DR [6,14,15]. Although animal studies have shown some benefits of various antioxidants such as vitamin E, SOD, Mito Q and lipoic acid on the oxidative stress related diseases [16,17], these agents have been proven to be partly effective in clinic. Vitamin E or other antioxidants appears to be limited to scavenge already-formed oxidants without inhibiting new ROS production [18,19]. In addition, these agents cannot reach the sites of free radical generation within the mitochondria. Given these limitations, it is understandable why antioxidant therapy remains an interesting yet unsatisfactory treatment for DR.

SS31, a small aromatic-cationic mitochondria-targeted peptide, can be selectively concentrated in the inner mitochondrial membrane and reduce mitochondrial ROS generation [7,10,20,21]. These suggest that SS31 can potentially be an excellent therapeutic candidate for treating DR. Our study demonstrated that 100 nM SS31 can effectively preserve HRECs cell viability and inhibit the mitochondrial ROS production induced by high glucose.

To evaluate the protective effect of SS31 on mitochondria function, we further measured mitochondrial potential and the release of cytochrome c from mitochondria into cytoplasm. Our data showed a rapid loss of $\Delta\Psi_m$ in HRECs and a marked release of cytochrome c in high glucose condition. These are in agreement with others previous studies [22–24]. But when treated with SS31, $\Delta\Psi_m$ deterioration and release of cytochrome c were restored. Alteration of $\Delta\Psi_m$ is an initial event during oxidative stress [25,26] indicating the dysfunction of mitochondria, whereas release of cytochrome c implies a disruption of the outer mitochondrial membrane. So our study suggests that SS31 can attenuate the damage of mitochondrial function from oxidative stress.

The combination of increased ROS and cytochrome c released into cytoplasm is able to activate pro-apoptotic caspase proteins which can trigger apoptosis. As a terminal factor in the enzymatic cascade reaction related to apoptosis, caspase-3 is an important effector leading to cell death [27]. Compared with that of the high glucose group, the expression of caspase-3 was reduced by 33.3% and 50.5% in the high glucose co-treated with SS31 at 24 and 48 h, respectively. Thus, suppression of caspase-3 expression may have contributed to the improved survival rate of HRECs in co-treatment with SS31 group in this study.

Another interesting discovery from this study is the up-regulation of Trx-2 in HRECs when co-treated with SS31. Thioredoxin-2 (Trx-2) is a mitochondria-specific member of the thioredoxin (Trx) super family. Trx, including Trx-1 and Trx-2, is universally expressed in the endothelial cells and can protect cells from H_2O_2-induced cytotoxicity [28]. Trx-1 is found in the cytosol, whereas Trx-2 is localized in the mitochondria. Overexpression of Trx-2 can protect cells against etoposide-induced apoptosis as well as increase mitochondrial membrane potential [27,29,30]. On the other hand, cells deficient in Trx-2 display increased cellular ROS and apoptosis [31]. Our previous study demonstrated that Trx-2 plays a critical role in preserving vascular endothelial cell function and preventing atherosclerosis development by reducing oxidative stress and increasing nitric oxide bioavailability [32]. This study suggests that in addition to reducing mitochondrial ROS production, SS31 may exert its cytoprotective function through upregulating the expression of the endogenous antioxidant Trx-2 thereby attenuating high glucose-induced injuries on HRECs.

All together, SS31 can attenuate high glucose-induced injuries on HRECs by stabilizing $\Delta\Psi_m$, decreasing ROS production, preventing the release of cytochrome c from mitochondria, decreasing the expression of caspase-3 and increasing the expression of Trx-2. SS31 may be as a potential new treatment for DR and other oxidative stress related diseases.

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