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Original contribution

Exendin-4 protects adipose-derived mesenchymal stem cells from apoptosis induced by hydrogen peroxide through the PI3K/Akt–Sfrp2 pathways

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ABSTRACT

Adipose-derived mesenchymal stem cells (ADMSCs)-based therapy is a promising modality for the treatment of myocardial infarction in the future. However, the majority of transplanted cells are readily lost after transplantation because of hypoxia and oxidative stress. An efficient means to enhance the ability of ADMSCs to survive under pathologic conditions is required. In our study, we explored the effects of exendin-4 (Ex-4) on ADMSCs apoptosis in vitro induced by hydrogen peroxide, focusing in particular on mitochondrial apoptotic pathways and PI3K/Akt–secreted frizzled-related protein 2 (Sfrp2) survival signaling. We demonstrated that ADMSCs subjected to H_2O_2 for 12 h exhibited impaired mitochondrial function and higher apoptotic rate. However, Ex-4 (1–20 nM) preconditioning for 12 h could protect ADMSCs against H_2O_2-mediated apoptosis in a dose-dependent manner. Furthermore, Ex-4 pretreatment upregulated the levels of superoxide dismutase and glutathione as well as down-regulating the production of reactive oxygen species and malondialdehyde. Western blots revealed that increased antiapoptotic proteins Bcl-2 and c-IAP1/2 as well as decreased proapoptotic proteins Bax and cytochrome *c* appeared in ADMSCs with Ex-4 incubation, which inhibited the caspase-9-involved mitochondrial apoptosis pathways with evidence showing inactivation of caspase-9/3 and preservation of mitochondrial membrane potential. Furthermore, we illustrated that Ex-4 enhanced Akt phosphorylation, which increased the expression of Sfrp2. Notably, blockade of the PI3K/Akt pathway or knockdown of Sfrp2 with siRNA obviously abolished the protective effects of Ex-4 on mitochondrial function and ADMSCs apoptosis under H_2O_2. In summary, this study confirmed that H_2O_2 induced ADMSCs apoptosis through mitochondria-dependent cell death pathways, and Ex-4 preconditioning may reduce such apoptosis of ADMSCs through the PI3K/Akt–Sfrp2 pathways.

Introduction

Although there have been many therapeutic advances in myocardial infarction (MI), irrevocable muscle damage and cardiomyocyte death contribute to the permanent loss of myocytes, leading to pathological left-ventricle remodeling and progression to cardiac dysfunction. Accumulating evidence during the past decade has revealed adipose-derived mesenchymal stem cells (ADMSCs) as a promising candidate to regenerate injured tissue via differentiation into cardiomyocytes [1] and the paracrine action of cytokines [2]. However, most transplanted cells undergo apoptosis within days after transplantation, which significantly reduces their effectiveness in tissue repair and compromises the optimal outcome of cell-based therapy for MI [3]. The primary factors in cellular apoptosis in ischemic heart are ischemia and oxidative stress [4], which induce a burst of excessive production of reactive oxygen species (ROS) not only during the reperfusion period [5] but also during the ischemic period [6], leading to impaired cell biology and decreased cell viability [7]. Therefore, protection of these cells from apoptosis, together with enhancing their ability to survival under ischemic or oxidative stress conditions, may be crucial for improving the efficiency of cell therapy [8].

Exendin-4 (Ex-4), isolated from the saliva glands of the Gila monster, shares a 53% homology with human glucagon-like peptide-1 (GLP-1) and plays a key role in the reduction of hyperglycemia by acting on the GLP-1 receptor (GLP-1R) [9]. It mimics the function of GLP-1 and exerts proliferation or survival promotion effects on many types of cells [10,11] via GLP-1R-dependent and -independent pathways [12,13]. However, little literature involves the role of Ex-4 in

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ADMSCs apoptosis under conditions of oxidative stress. Our previous studies [14,15] have found that Ex-4 pretreatment could prevent hydrogen peroxide (H_2O_2)-induced apoptosis of ADMSCs via activation of Akt, but the mechanism and downstream signal of Akt remain unknown. Recently, secreted frizzled-related protein 2 (Sfrp2) was identified as a main downstream executive of the PI3K/Akt signaling pathway on mesenchymal stem cell (MSC) survival [16]. However, whether Sfrp2 could be activated by Ex-4 via PI3K/Akt and whether the PI3K/Akt–Sfrp2 pathways are necessary for the antiapoptotic effect of Ex-4 on ADMSCs are far from clear.

In this study, H_2O_2 was used to induce ADMSCs apoptosis with or without pretreatment with Ex-4. The results indicated that exogenously added H_2O_2 could induce apoptosis in ADMSCs through mitochondrial death pathways, and Ex-4 partially abolished such effect of H_2O_2 in a dose-dependent manner. Furthermore, we provided evidence that the PI3K/Akt–Sfrp2 pathways were required for the antiapoptotic action of Ex-4 on ADMSCs by reducing mitochondrial oxidative stress injury.

Materials and methods

This study was performed in accordance with the Declaration of Helsinki and the guidelines of the Ethics Committee of the Chinese PLA (People's Liberation Army) General Hospital, Beijing, China

ADMSCs culture, differentiation, characterization, and model of oxidative stress injury

ADMSCs were isolated from inguinal adipose tissue of Sprague–Dawley rats (60–80 g, obtained from the Laboratory Animal Center, Chinese PLA General Hospital) as previously described [17]. Briefly, subcutaneous fat tissue from the inguinal region was washed with PBS (phosphate-buffered saline) three times and then digested with 0.1% collagenase II (Sigma–Aldrich, St. Louis, MO, USA) and 0.05% trypsin (Sigma–Aldrich) for 45 min at 37 °C with continuous shaking. After addition of Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich), the samples were filtered through a 70-nm metal mesh filter followed by centrifugation for 10 min at 400*g* to obtain the stromal cell fraction. The supernatant was discarded and the pellet was resuspended in DMEM supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 units/ml penicillin, and 100 mg/ml streptomycin. The cells were cultured at 37 °C/5% CO_2 and the culture medium was replaced every 3 days. The experiments were undertaken with ADMSCs in the fourth or fifth passage.

For adipogenic differentiation, adipogenic medium (DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μmol/L L-ascorbic acid, 1 μmol/L dexamethasone, 0.5 mmol/L 1-methyl-3-isobutylxanthine, and 100 μmol/L indomethacin) was used. Osteogenic differentiation was conducted by incubating cells at 100% confluence on coverslips with DMEM supplemented with 10% FBS, 0.1 μmol/L dexamethasone, 200 μmol/L L-ascorbic acid, and 10 mmol/L β-glycerol phosphate. After culture for 3–4 weeks, adipogenesis was assessed by incubating cells with oil red O solution to stain neutral lipids in the cytoplasm. To assess mineralization, calcium deposits were stained with Alizarin Red S (Sigma–Aldrich).

ADMSCs at passage 4 were gathered to detect surface antigens using flow cytometry analysis. After being washed in PBS, ADMSCs were incubated with anti-rat monoclonal fluorescent antibody, FITC-labeled CD29, CD31, CD34, CD45, CD90 (BD Biosciences, San Jose, CA, USA) or GLP-1R (Bioss, Shanghai, China) or FITC-labeled IgG at the manufacturer-recommended concentration for 30 min in the dark. Flow cytometric analyses were performed on a BD FACSCalibur cytometer.

Apoptosis was induced by hydrogen peroxide and serum deprivation. In brief, after cells were washed with PBS, the culture medium was replaced with serum-free DMEM supplemented with 0.3 mM H_2O_2 and then the cells were placed at 37 °C under normoxic conditions for 12 h. For Ex-4 protection experiments, ADMSCs were pretreated with Ex-4 (Sigma–Aldrich, 0–20 nM) for 12 h and then continuously treated with 0.3 mM H_2O_2 for 12 h.

Effects of Ex-4 on cell viability

To assess whether Ex-4 itself could affect cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma–Aldrich) was used to examine cell viability under various concentrations of Ex-4 (0–20 nM). Cells were seeded in 96-well plates with Ex-4 in triplicate. Then 20 μl of MTT (5 mg/ml PBS, pH 7.4) was added for another 4 h. The supernatant was then discarded and 100 μl of dimethyl sulfoxide was added into each well for 10 min. Finally, the optical density was measured at $A_{490\ nm}$.

Measurement of markers of oxidative damage and antioxidant capacity of ADMSCs

ROS, a characterization of oxidant status, are involved in the injury of ADMSCs in the setting of oxidation stress. Dihydroethidium (DHE; Invitrogen, San Diego, CA, USA) was used to detect the intracellular ROS and 10 μM DHE was added to the cell culture medium, which was then incubated in the dark and viewed under laser confocal microscopy (Olympus). Malondialdehyde (MDA), an end product of peroxidation of cell membrane lipids caused by oxidative free radicals, is considered a reliable marker of oxidative damage. Glutathione (GSH) and superoxide dismutase (SOD) play important roles in clearing ROS, thereby protecting cells from oxidative damage. The MDA content, SOD activity, and GSH concentration were measured using commercial kits (Beyotime Institute of Biotechnology, Shanghai, China) following the manufacturer's instructions.

Assessment of morphological changes of apoptosis

The chromatin dye Hoechst 33342 (Beyotime Institute of Biotechnology) was used for the detection of nuclear fragmentation and condensation, which were identified as the morphological alterations of apoptotic cells. After treatment, the ADMSCs were fixed at room temperature for 15 min in PBS containing 4% paraformaldehyde and then stained with Hoechst 33342 (5 μg/ml) for 30 min in the dark. All samples were observed under fluorescence microscopy. Apoptotic cells were identified as having condensed, fragmented nuclei and undergoing cell shrinkage.

Flow cytometry analysis of cell apoptosis and mitochondrial membrane potential ($\Delta\Psi m$)

An annexin V–FITC/PI apoptosis detection kit (BD Biosciences) was used to quantitatively analyze the number of apoptotic cells. Briefly, cells were harvested and resuspended in 200 μl binding buffer after being incubated with 5 μl annexin V–FITC solution (30 min, 37 °C) in the dark. The cells were then incubated with 10 μl propidium iodide for 5 min and immediately analyzed by bivariate flow cytometry using BD FACSCalibur cytometry.

The mitochondrial transmembrane potential was analyzed using a mitochondrial membrane potential detection kit (JC-1)

(Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Briefly, cells were washed with ice-cold PBS and then stained with 2.5 g/ml JC-1 for 30 min at 37 °C. After being washed with binding buffer, the cells were analyzed by FACScan or fluorescence microscopy. Results are presented as relative aggregate-to-monomer (red/green) fluorescence intensity ratio.

Western blot analysis

For analysis of protein levels, stimulated cells were rinsed twice with ice-cold PBS and then lysed in RIPA buffer containing protein inhibitor for 30 min. Equal amounts of proteins were loaded on 8–15% SDS–PAGE gels and then transferred to polyvinylidene difluoride membranes (Sigma–Aldrich). The membranes were incubated with 5% nonfat milk for 2 h at room temperature followed by primary antibody in 5% nonfat milk overnight at 4 °C (β-actin, 1:2000; caspase-3, 1:2000; procaspase-3, 1:1000; caspase-9, 1:1000; t-Akt, 1:2000; p-Akt, 1:1500; and c-IAP1, 1:1000; purchased from Cell Signaling Technology, Danvers, MA, USA; c-IAP2, 1:1000; cytochrome *c*, 1:2000; Bcl-2, 1:2000; Bax, 1:2000; and Sfrp-2, 1:1000; purchased from Abcam, Cambridge, MA, USA). After being washed in TBST for 30 min, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for 45–60 min at room temperature. Bands were visualized by enhanced chemiluminescence reagent (Beyotime Institute of Biotechnology) after the membranes were washed with TBST (10 min × 3).

Immunofluorescence staining

To determine cytochrome *c* (Cyt *c*) localization, immunofluorescence staining was used. In brief, cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.3% Triton X-100 for 5 min, and blocked with 10% goat serum albumin (Invitrogen) for 1 h at room temperature. Specimens were subsequently incubated with primary antibodies against Cyt *c* (Abcam) overnight at 4 °C, then washed with PBS three times, and incubated with Alexa Fluor 488 donkey anti-rabbit secondary antibody (Invitrogen) for 45 min at room temperature. After being washed, the cells were treated with 4′,6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich) for 5 min and analyzed under a fluorescence microscope.

Terminal deoxynucleotidyl transferase-mediated dUTP–biotin nick-end labeling (TUNEL) assay

TUNEL assay was used to detect the apoptosis of ADMSCs according to the manufacturer's protocol. TUNEL staining was performed with fluorescein–dUTP (Invitrogen) for apoptotic cell nuclei and DAPI to stain all cell nuclei. Cells in which the nucleus was stained were defined as TUNEL positive.

Determination of caspase activity

Because activation of caspase-3 represents an essential step in the apoptotic process, a caspase-3 activity kit (Beyotime Institute of Biotechnology) was used to detect the activity of caspase-3 according to the manufacturer's protocol. Briefly, cells were lysed after treatment with various designated interventions followed by collection and quantification of protein concentration in the supernatants using a BCA protein assay. After normalization, 10 μl of cell lysate protein, 80 μl of reaction buffer (1% NP-40, 20 mM Tris–HCl, pH 7.5, 137 mM NaCl, and 10% glycerol), and 10 μl of caspase-3 substrate (Ac-DEVD-pNA; 2 mM) per sample were added into 96-well microtiter plates in a triple pattern. Ninety microliters of reaction buffer and 10 μl of caspase-3 substrate were used as the negative group. After incubation at 37 °C for 4 h, samples were measured with an ELISA reader at an absorbance of 405 nm. Relative caspase-3 activity was calculated as the ratio of emission of treated cells to emission of untreated cells (normal cells). The assay was repeated three times

Lactate dehydrogenase release

Lactate dehydrogenase (LDH) is a fairly stable enzyme that is released from the cytosol into the culture medium as a consequence of cellular integrity damage from H_2O_2. Thus, we used an LDH assay (Beyotime Institute of Biotechnology) to evaluate the presence of cell injury or damage. The level of LDH released was expressed as a percentage of the control (normal cells) group.

RNA interference (RNAi) knockdown

The small interfering RNA (siRNA) targeting Sfrp2 was designed as follows: $siRNA_1$ sense, 5′-GAGGAUGACAACGACAUAATT-3′, antisense, 5′-UUAUGUCGUUGUCAUCCUCTT-3′; $siRNA_2$ sense, 5′-UCUCCUACAAGCGCAGCAATT-3′, antisense, 5′-UUGCUGCGCUUGUAGGAGATT-3′; and negative control $siRNA_{ctrl}$ sense, 5′-UUCUCCGAACGUGUCACGUTT-3′; antisense, 5′-ACGUGACACGUUCGGAGAATT-3′. These were designed and synthesized by Shanghai Gene-Pharma Co. (Shanghai, China). For the RNAi knockdown, cells were seeded in plates containing medium without antibiotics for 24 h before transfection. The siRNAs were transfected into the cells using Lipofectamine 2000 (Invitrogen) in serum-free Opti-MEM (Invitrogen), according to the manufacturer's instructions. The expression levels of Sfrp2 in transfected cells were determined by Western blot analysis. The cells were harvested at the indicated time points and used for further analysis

Statistical analysis

Data are expressed as means ± standard deviation. Comparisons between two groups were measured using Student's *t* test. Differences among groups were detected by one-way ANOVA. A value of $p < 0.05$ was considered significantly different.

Results

Characterization of cultured ADMSCs

ADMSCs cultured in medium displayed a spindle-shaped or fibroblast-like morphology (Fig. 1A). To establish the multilineage differentiation ability of ADMSCs, cells were incubated in adipogenic or osteogenic medium for 3–4 weeks. The results in Fig. 1A indicate that ADMSCs could differentiate into adipogenic and osteogenic cells. Meanwhile, the cells at passage 4 were positive for CD29 (87.30%) and CD90 (92.49%) by flow cytometry analysis. Additionally, the cells were negative for the CD31 (2.30%), CD34 (0.03%), and CD45 (0.04%), suggesting that the cell populations were not contaminated with hematopoietic stem cells or endothelial cells (Fig. 1B).

Effects of Ex-4 on cell viability and the expression of GLP-1R on ADMSCs

First, we evaluated whether Ex-4 itself could affect cell viability. As shown in Fig. 2A, at the concentrations used (1–20 nM), Ex-4 had little impact on cell viability compared with normal cells, indicating that Ex-4 had no toxic effects on ADMSCs.

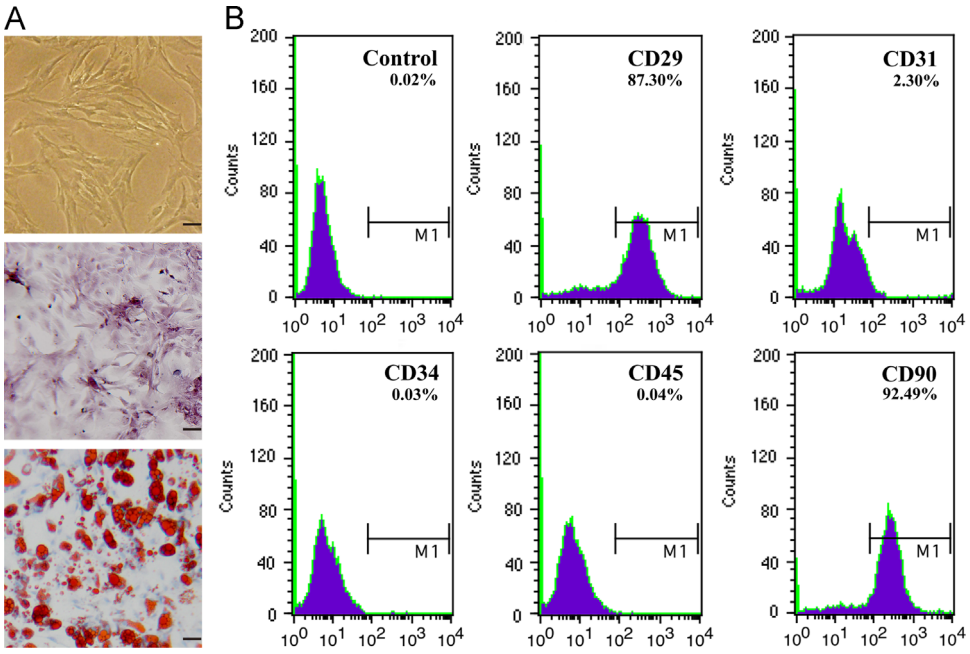

Fig. 1. Characterization of ADMSCs. (A) Isolated ADMSCs displayed fibroblast-like shapes and could differentiate into osteogenic and adipogenic lineages by Alizarin Red S and oil red O staining. (B) Flow cytometry results showed that ADMSCs were uniformly negative for CD31, CD34, and CD45 and positive for CD29 and CD90. Bar, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Ex-4, a GLP-1R agonist, exhibits a cell-protective effect on many types of cells via GLP-1R-dependent and -independent pathways [12,13]. Moreover, some studies have reported that Ex-4 treatment could alter the expression of GLP-1R [18,19]. Thus, we wanted to explore the expression of GLP-1R on ADMSCs with or without Ex-4. As shown in Fig. 2B, GLP-1R expression was low to undetectable on the ADMSCs surface. Although there was a very small trend toward increased expression of GLP-1R after Ex-4 (1–20 nM) treatment, the difference did not reach significance. Thus, the experimental results showed that little expression of GLP-1R was observed in ADMSCs regardless of incubation with or without Ex-4, which is consistent with previous studies [20,21]. These data indicated that the effect of Ex-4 on ADMSCs may be mediated via a GLP-1R-independent pathway.

Roles of Ex-4 in H_2O_2-induced cell apoptosis

To examine the effects of Ex-4 on H_2O_2-induced cell apoptosis, ADMSCs were pretreated with Ex-4 (0–20 nM) for 12 h before being exposed to 0.3 mM H_2O_2 for 12 h, and the number of apoptotic cells was evaluated by flow cytometry. Under our experimental conditions, approximately $3.47 \pm 2.74\%$ of cells in the control group were annexin V^+/PI^- and H_2O_2 markedly increased annexin V^+/PI^- cells ($36.80 \pm 7.93\%$, $p < 0.05$ vs control). However, when the ADMSCs were pretreated with Ex-4 for 12 h before H_2O_2, the number of annexin V^+/PI^- cells significantly decreased in a concentration-dependent manner (Ex-4 1 nM, $14.14 \pm 3.91\%$; 5 nM, $12.34 \pm 3.62\%$; 10 nM, $11.62 \pm 3.42\%$; 20 nM, $6.41 \pm 3.08\%$; $p < 0.05$ vs H_2O_2; Fig. 3A), suggesting that Ex-4 could abolish the effects of H_2O_2 in the early apoptosis phase (annexin V^+/PI^-). However, Ex-4 had no effects on the late apoptosis phase (annexin V^+/PI^+; $p>0.05$ vs H_2O_2) or necrosis (annexin V^-/PI^+; $p>0.05$ vs H_2O_2). Because the above results indicated that Ex-4 at concentrations of 1, 5, 10, and 20 nM had equipotent antiapoptotic effects, a concentration of 20 nM was used for the following experiments.

The antiapoptotic effects of Ex-4 were further confirmed qualitatively by morphological changes with Hoechst 33342. Unlike the rod-shaped morphology of the normal ADMSCs (Fig. 3B), rounded or irregular shape, cell shrinkage, and chromatin condensation were observed in ADMSCs exposed to H_2O_2. However, preconditioning with 20 nM Ex-4 for 12 h prevented these morphological changes.

Because excessive ROS are the determining pathogenic factor in ADMSCs apoptosis in the setting of oxidative stress, we wanted to explore the effect and mechanism of Ex-4 on ROS levels in ADMSCs. As shown in Fig. 3B, H_2O_2 increased the intracellular ROS levels but Ex-4 inhibited the accumulation of ROS. In parallel, the MDA concentration was significantly increased in ADMSCs with H_2O_2 and Ex-4 could reverse this trend. In addition, the activities of SOD and GSH were increased in the Ex-4 group compared to the H_2O_2 groups. SOD and GSH are the most important intracellular antioxidant factors that exert a key role in counteracting and clearing ROS [22]. These results indicated that Ex-4 plays a vital role in enhancing the antioxidant defense system in ADMSCs.

Ex-4 protects mitochondrial function in H_2O_2-induced ADMSCs apoptosis

Excessive exogenous ROS could attack mitochondria causing reduced electrochemical gradient ($\Delta\Psi m$) across mitochondria membranes [23]. Furthermore, loss of $\Delta\Psi m$ results in the permeability transition pore opening and release of mitochondrial contents (such as Cyt *c*) into the cytoplasm [24], ultimately leading to the execution phase of apoptosis, which is identified as the mitochondrial death pathway [25,26]. Therefore we postulated that H_2O_2, a member of the ROS, induced apoptosis in ADMSCs through the mitochondrial death pathway and that the antiapoptotic effect of Ex-4 on ADMSCs under H_2O_2 was depended on preserving mitochondrial function. First, JC-1, a mitochondrial $\Delta\Psi m$-sensitive dye, was used to measure the mitochondrial transmembrane potential. Normal cells stained with JC-1 exhibited red fluorescence indicative of coupled mitochondria with a normal $\Delta\Psi m$. However, when the $\Delta\Psi m$ was low, JC-1 became a monomer with green fluorescence. In our flow cytometry analysis, the R_2 and R_3 regions represented distribution of green and red fluorescence, respectively. The results

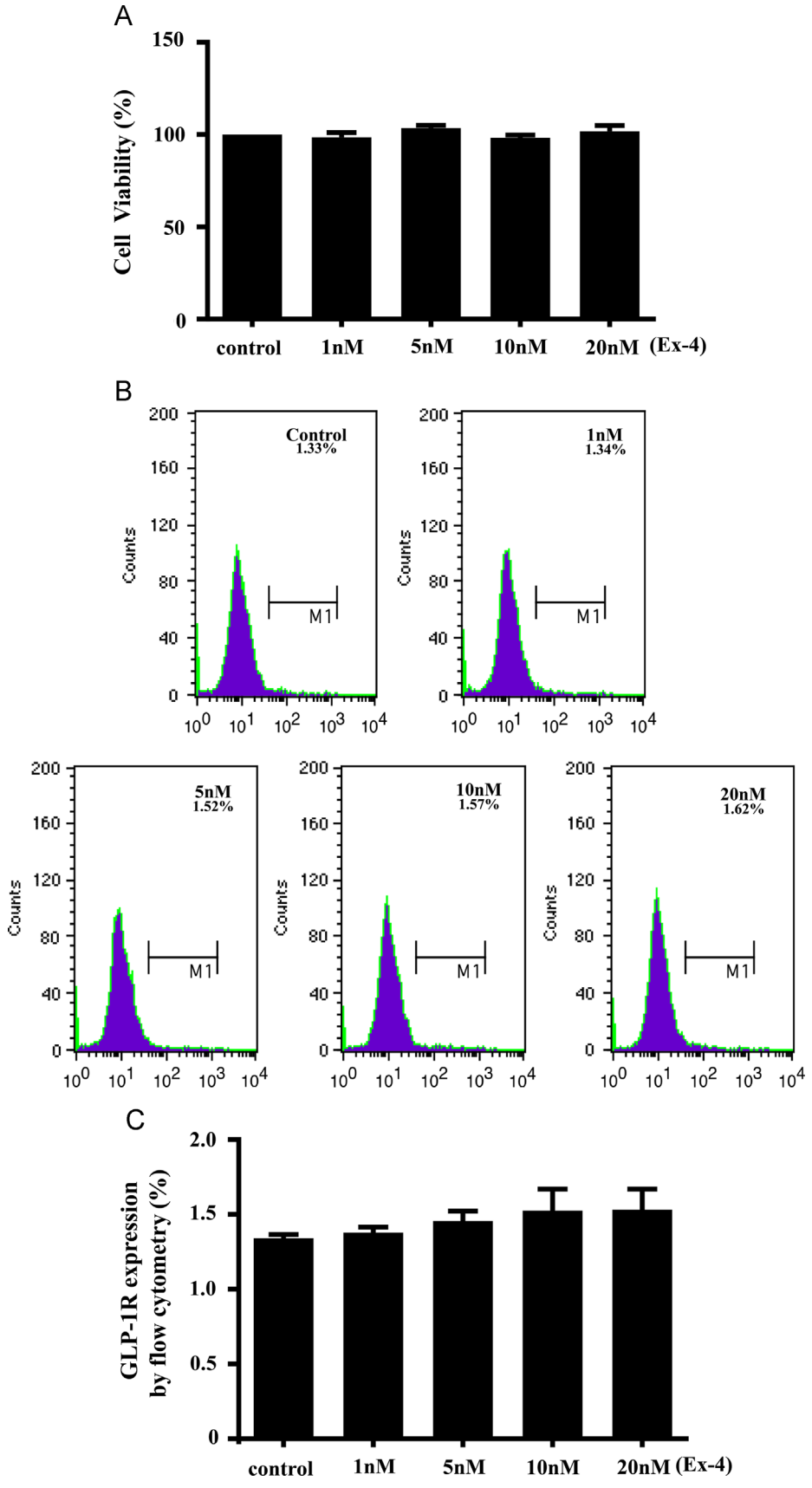

Fig. 2. Effects of Ex-4 on cell viability and GLP-1R expression. (A) Ex-4 had no cytotoxic effect on ADMSCs. (B and C) Expression of GLP-1R on the ADMSCs surface.

obtained revealed that H_2O_2 caused marked changes in mitochondrial $\Delta\Psi m$ compared with the control group. However, Ex-4 pretreatment maintained an electrochemical gradient across the mitochondrial membranes in a concentration-dependent manner (Figs. 4A and B).

Leakage of Cyt *c*, as the consequence of reduced $\Delta\Psi m$, is a terminal event during mitochondria-related apoptosis [27]. Hence, we used immunostaining to investigate Cyt *c* release under H_2O_2-induced apoptosis. The results revealed that Cyt *c* in normal ADMSCs was characterized by a punctate appearance in mitochondria. However, treatment of ADMSCs with H_2O_2 resulted in diffusion of Cyt *c* from mitochondria into cytoplasm (Fig. 4C), but 20 nM Ex-4 preconditioning partly inhibited such effect.

Released Cyt *c* triggers apoptosome formation (a complex comprising Apaf-1, Cyt *c*, dATP, and procaspase-9) and caspase-9 activation that in turn activates procaspase-3 to form the death effector cleaved caspase-3 [28]. Therefore, Western blots were used to further examine Cyt *c* and caspase-9/3 expression in cytoplasm. As shown in Fig. 4D, H_2O_2 caused significantly increase in Cyt *c* and caspase-9/3 and Ex-4 could inhibit such effects of H_2O_2. Meanwhile, we found that Ex-4 pretreatment suppressed proapoptotic protein Bax as well as upregulating antiapoptotic proteins Bcl-2 and c-IAP1/2 under H_2O_2. It is known that the balance of Bcl-2 and Bax regulates the stabilization of $\Delta\Psi m$ and mitochondrial function [29] and that c-IAP1/2 have a role in suppressing Cyt *c* release and caspase-9 activation [30], which ultimately preserves mitochondrial function and inactivates caspase-9/3 [31]. The data altogether suggest that Ex-4 is of importance in the protection of mitochondrial function through recruitment of antiapoptotic proteins that block mitochondrial death pathways under H_2O_2-induced apoptosis.

Sfrp2 is activated by Ex-4 via the PI3K/Akt pathway and contributes to Ex-4's antiapoptotic effect

Our previous studies have found that Ex-4 is an upstream activator of the PI3K/Akt signaling pathway [14], which is involved

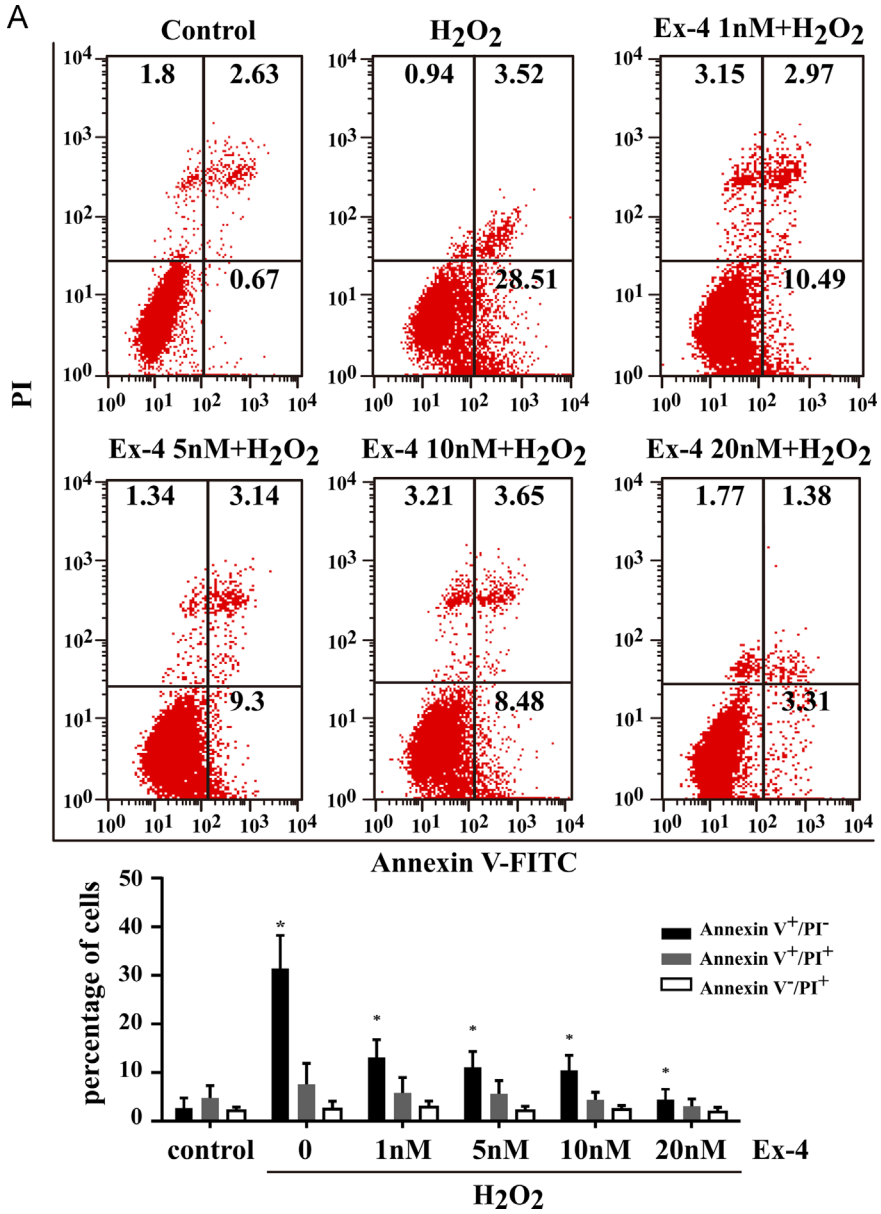

Fig. 3. Antiapoptotic actions of Ex-4 on ADMSCs after oxidative stress injury. Apoptosis was determined quantitatively by flow cytometry using an annexin V/PI kit and analyzed qualitatively with Hoechst 33342 staining. (A) Annexin V/PI assay. (B) Hoechst 33342 staining. (C–F) The changes in ROS, MDA, GSH, and SOD levels. Cell nuclei are blue. $^*p < 0.05$ vs control group or H_2O_2 group.

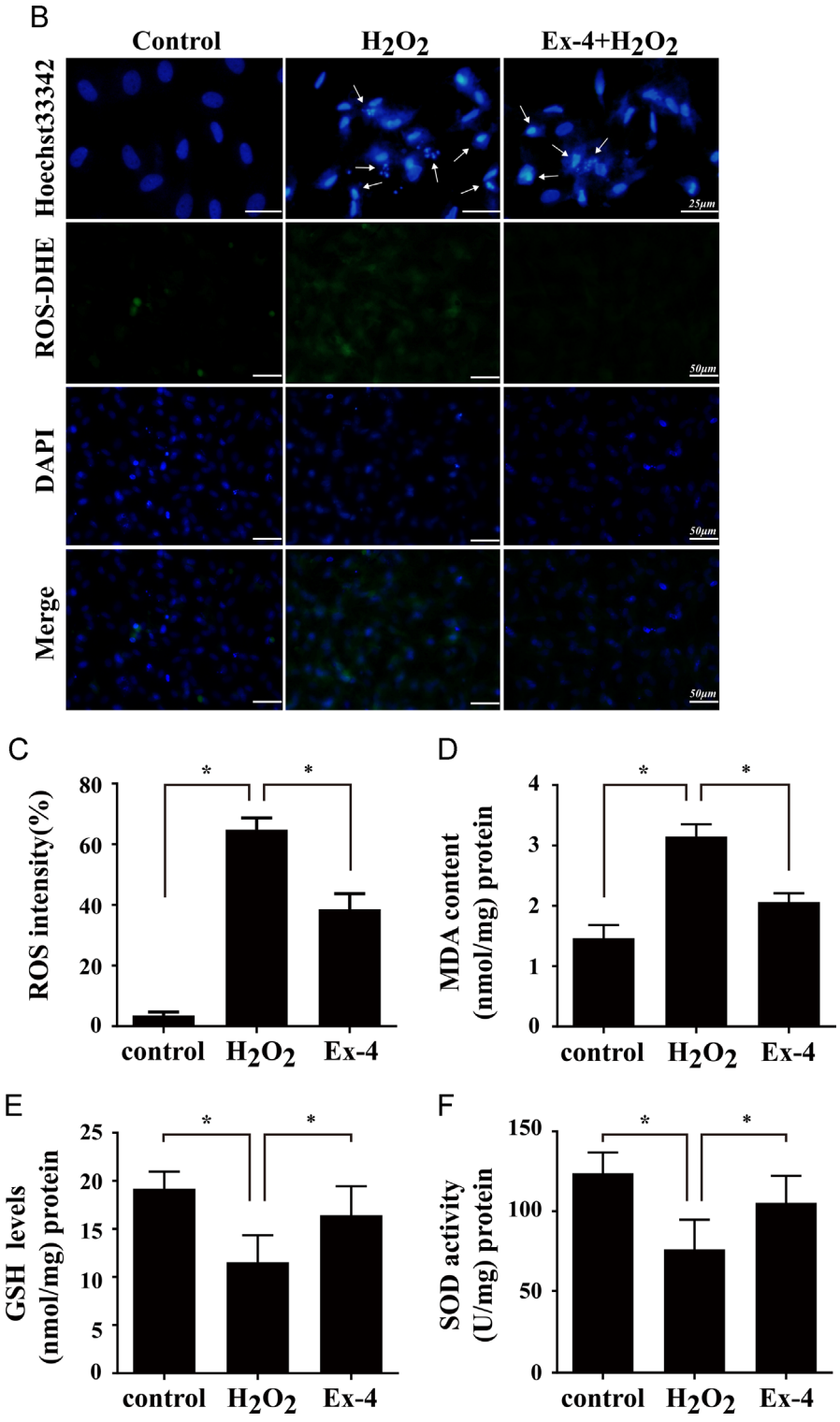

Fig. 3. (*continued*)

in the transduction of antiapoptotic signals in various cells under oxidative injury [32,33], and Sfrp2 has been identified as the major downstream mediator of the PI3K/Akt pathway in MSC survival under low-oxygen conditions [34,35]. We therefore speculated whether the capability of Ex-4 to recruit antiapoptotic proteins and protect ADMSCs from apoptosis was attributable to Sfrp2. To test our hypothesis, we first examined the expression changes in the PI3K/Akt pathway and Sfrp2 with Ex-4 preconditioning. As shown in Fig. 5A, exposure of ADMSCs to Ex-4 increased the levels of phosphorylated Akt (p-Akt) and Sfrp2, but the PI3K/Akt pathway inhibitor LY294002 potently blocked the Ex-4-mediated increment in Sfrp2, indicating that Ex-4-induced upregulation of Sfrp2 in ADMSCs depends on the PI3K/Akt pathway.

To further elucidate whether Ex-4's prosurvival signal was Sfrp2 dependent, an RNAi method was used and knockdown of Sfrp2 with two independent siRNAs was assessed by Western blot. As shown in Fig. 5B, the protein expression of Sfrp2 was significantly reduced in ADMSCs transfected with siRNA against Sfrp2

but not by control siRNA. Furthermore, knockdown of Sfrp2 eliminated the protective effects of Ex-4 on mitochondria, leading to loss of $\Delta\Psi m$ and Cyt *c* release (Figs. 5C and D). Importantly, inhibition of Sfrp2 with siRNA abolished Ex-4-mediated upregulation of antiapoptotic proteins but increased proapoptotic proteins (Fig. 5E). These data suggested that Ex-4-induced recruitment of antiapoptotic proteins was attributable to Sfrp2 which protected mitochondrial function from oxidative injury.

The PI3K/Akt pathway is involved in Ex-4's antiapoptotic effect

We have demonstrated that Ex-4 regulates Sfrp2 expression via the PI3K/Akt pathway and Sfrp2 is a key mediator of Ex-4's antiapoptotic effect. To investigate whether the PI3K/Akt pathway is also involved in the survival signal of Ex-4 in ADMSCs with H_2O_2, a pathway blocker of PI3K/Akt was used. As shown in Figs. 6A and B, H_2O_2-induced ADMSCs apoptosis showed evidence of more $TUNEL^+$ cells and higher LDH release, and few $TUNEL^+$ cells as well as low LDH contents were revealed in the Ex-4 group. However, pretreatment of ADMSCs with LY294002 significantly abolished the Ex-4 protective effects on ADMSCs apoptosis. Because caspase-3-mediated protein shear is a key factor in cell apoptosis, we explored the caspase-3 activity under Ex-4 intervention when PI3K/Akt was inhibited or not. Our data demonstrated that under H_2O_2 treatment, compared with the H_2O_2 group, Ex-4 treatment resulted in a significant decrease in caspase-3 activity, which was inhibited by LY294002. These results revealed that the PI3K/Akt pathway also contributed to the antiapoptotic effect of Ex-4 on ADMSCs.

Discussion

In the past decades, the use of ADMSCs transplantation therapy to compensate for the damaged myocardium has become increasingly popular in animal studies and clinical trials. Despite visible advances in the field of stem-cell-based therapy, the reported functional improvements are generally modest partly because of the low cellular survival rate [36] triggered by an ischemic environment in vivo [37]. Under ischemic conditions, the decrease in antioxidants and the increase in oxidative stress results in elevated levels of damaging ROS in the infarcted heart [38], which challenges the viability of the transplanted cells. Accordingly, improving the survival capacity of ADMSCs in oxidative injury is urgent, and several strategies have been introduced to enhance the tolerance of MSCs to apoptosis by genetic modification or by preconditioning cells with drugs that potentiate the longevity of engrafted cells in hostile environments [28,39]. However, the high cost, adverse side effects, and technical hurdles have limited their application. In our study, we used a new drug, Exendin-4, to pretreat ADMSCs before H_2O_2, and then observed the protective effects of Ex-4 on the ADMSCs apoptosis under oxidative injury conditions.

Ex-4, an antidiabetic agent, originally isolated from the venom of the Gila monster lizard [40], shares 53% amino acid sequence identity with human GLP-1 [41] and so is a potent stimulator of the GLP-1R [42]. Ex-4 exhibits biologic actions similar to those of GLP-1 and these effects are partly mediated by the activation of GLP-1R, which is widely expressed in various organs and tissues, including pancreatic islets, ductal cells, lung, pituitary, heart, and kidney [43]. After Ex-4 acts on cells, three main intracellular signal pathways (cAMP/PKA, PI3K/Akt, MAPK) were activated [44]. However, Ban et al. [12] first found that the actions of GLP-1 were preserved in $GLP\text{-}1R^{-/-}$ mice. Other studies have also demonstrated that Ex-4 could activate signal transduction pathways through a GLP-1R-independent pathway [45,46]. In addition, some experiments discovered that Ex-4 incubation could alter GLP-1R expression on the cell surface [18,19]. Our results displayed that there was almost no expression of GLP-1R on ADMSCs with or without Ex-4 treatment, which was consistent with previous studies [20,21], indicating that the effect of Ex-4 on ADMSCs is mediated via a GLP-1R-independent route. Tatarkiewicz et al. [47]

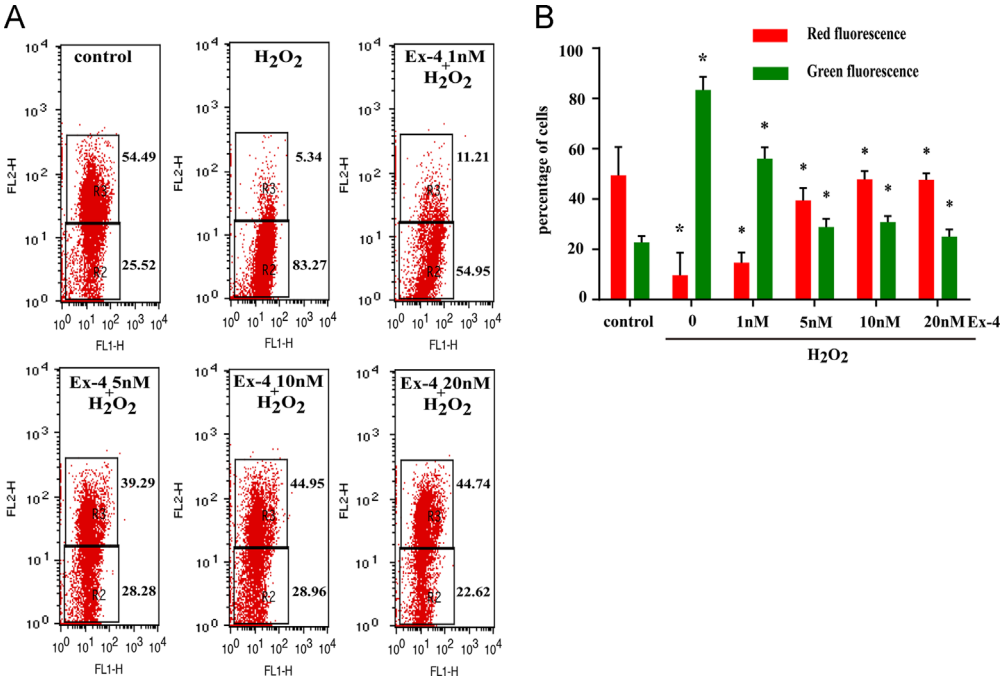

Fig. 4. Ex-4 protected mitochondrial function in H_2O_2-induced ADMSCs apoptosis. (A and B) The change in $\Delta\Psi m$ with Ex-4 before H_2O_2 by flow cytometry. (C) Immunostaining of ADMSCs for Cyt *c*. Typical punctate staining (green) for Cyt *c* was observed in ADMSCs without H_2O_2, which corresponded well with the location of mitochondria. However, the punctate appearance was located in cytoplasm after H_2O_2 treatment, whereas Ex-4 preconditioning inhibited such effects. (D) The change in protein expression in association with mitochondrial apoptosis pathways. Cell nuclei are blue. Scale bar, 100 μm. $^*p < 0.05$ vs control group or H_2O_2 group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

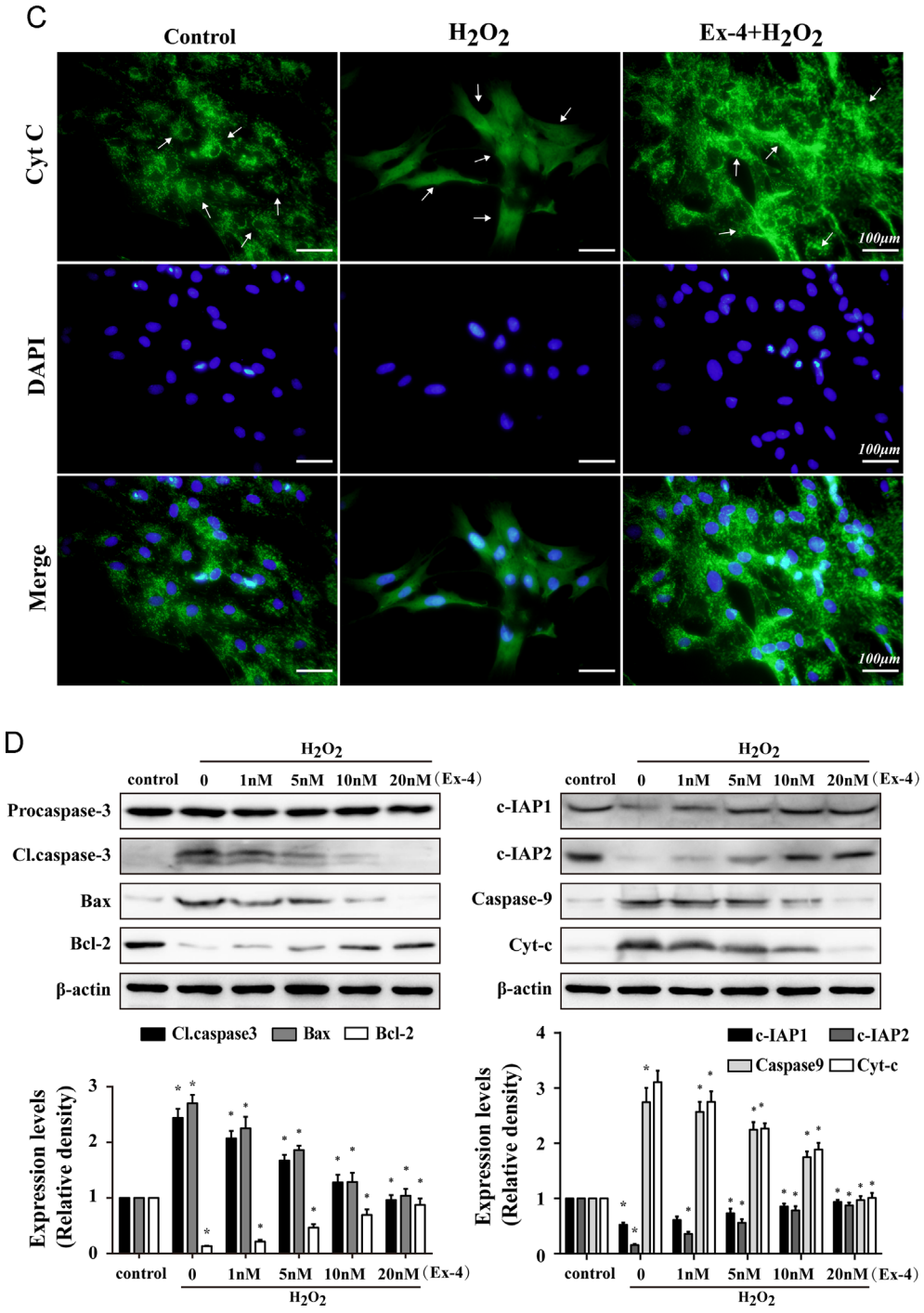

Fig. 4. (*continued*)

suggested the existence of an additional novel receptor signaling pathway complementing the actions of Ex-4. Kappe et al. [48] found the cross talk between tyrosine kinase receptors (e.g., the insulin receptor) and GLP-1R and that Ex-4 may exert an effect on tyrosine kinase receptors, which transmit signals to activate downstream pathways of GLP-1R. However, the exact mechanism of how Ex-4 enters into ADMSCs and subsequently activates signal pathways needs more research.

In our study, H_2O_2 application resulted in an increase in intracellular ROS and MDA as well as a decrease in GSH and SOD. However, Ex-4 pretreatment could reverse such effects of H_2O_2. MDA is produced from lipid peroxidation triggered by ROS, and the intracellular MDA levels reflect the degree and severity of oxidative injury. SOD and GSH are the most important intracellular antioxidants—they can eliminate excessive ROS and maintain normal redox levels in cells. Many studies have found that Ex-4 could directly clear ROS [49], whereas Eri Mukai argued that the Epac signaling pathway is involved in the ROS-scavenging effect of Ex-4 [50]. Our previous study suggested that Akt/STAT3 pathways may be required for Ex-4-induced ROS clearance [14]. In the present study, we revealed that Ex-4 played a role in enhancing the endogenous antioxidant defense system, which contributed to the reduction in intracellular ROS.

Our data found that H_2O_2 induced ADMSCs apoptosis through caspase-9-dependent mitochondrial death pathways, but Ex-4 pretreatment inhibited cellular apoptosis via preservation of

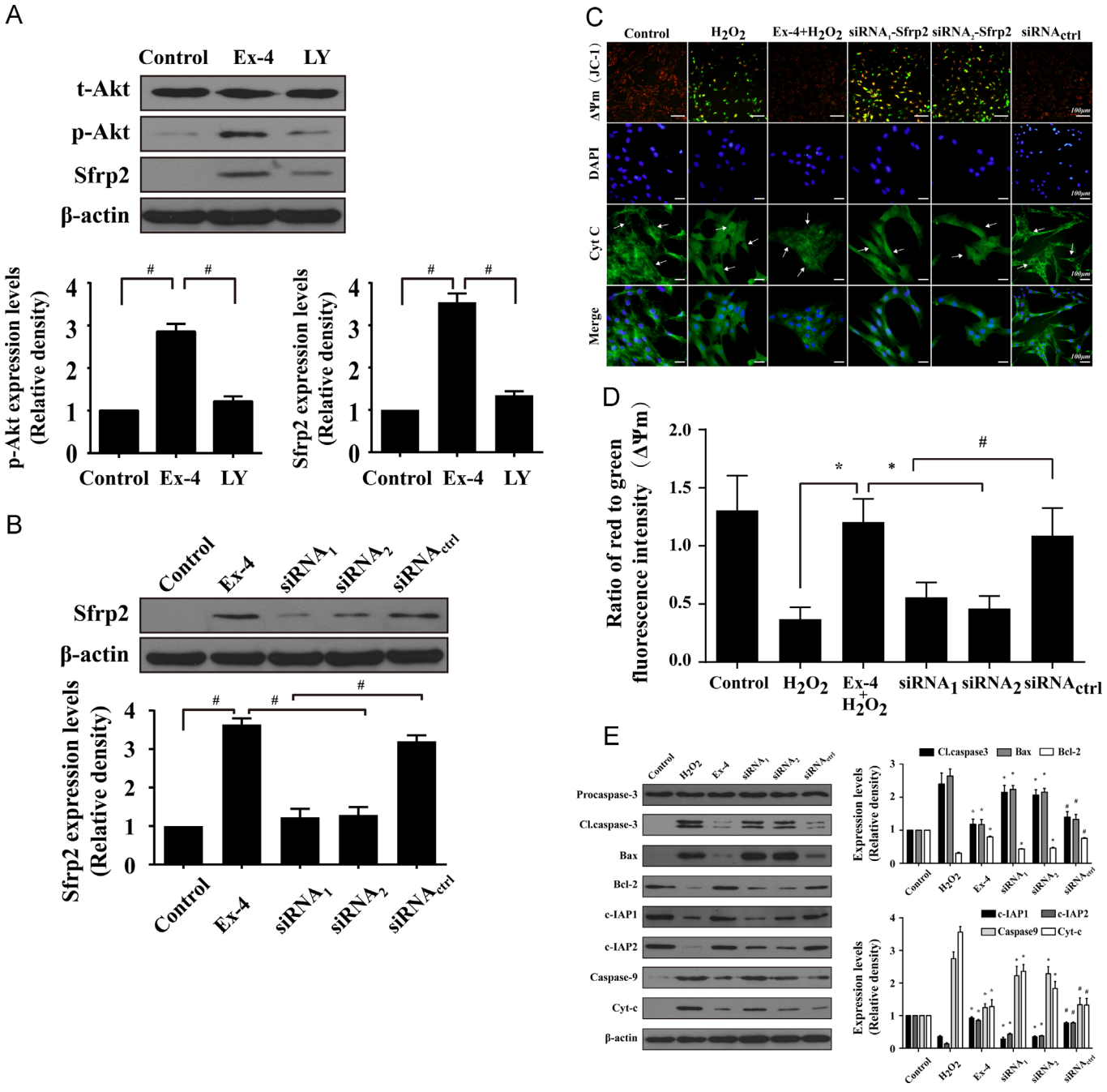

Fig. 5. Ex-4 improved the expression of Sfrp2, which was necessary for Ex-4's antiapoptosis effects. (A) Ex-4 induced the accumulation of Sfrp2 in ADMSCs through the PI3K/Akt pathway. $^{\#}p < 0.05$. (B) Relative reduction in protein level of Sfrp2 in ADMSCs after knockdown of Sfrp2 with siRNA. $^{\#}p < 0.05$. (C) Reduced ΔΨm and release of Cyt *c* were observed under fluorescence microscopy. (D) Quantitative analysis of the change in ΔΨm using the ratio of red to green fluorescence. (E) The protein changes after Sfrp2 were inhibited with siRNA. Cell nuclei are blue. Scale bar, 100 μm. LY, LY-294002; $siRNA_1$ and $siRNA_2$, ADMSCs transfected with two independent siRNAs against Sfrp2 and treated with Ex-4; $siRNA_{ctrl}$, ADMSCs transfected with control siRNA and treated with Ex-4. $^{*}p < 0.05$ vs H_2O_2 or Ex-4, $^{\#}p < 0.05$ vs $siRNA_1$ or $siRNA_2$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mitochondrial function, with evidence showing maintained ΔΨm and reduced Cyt *c* leakage. Meanwhile, our experiment demonstrated that H_2O_2 increased proapoptotic protein Bax levels but decreased anti-apoptotic proteins Bcl-2, c-IAP1, and c-IAP2. The balance of Bax and Bcl-2 is responsible for the integrity of the mitochondrial membrane and ΔΨm stabilization [27]. c-IAP1 and c-IAP2 are inhibitors of Cyt *c* and caspase-9. Higher Bax and lower Bcl-2 levels resulted in a loss of ΔΨm, Cyt *c* release, and mitochondrial swelling or disruption [51]. In contrast, Ex-4 pretreatment upregulated Bcl-2, c-IAP1, and c-IAP2 but downregulated Bax, which contributed to the preservation of mitochondrial function. In addition, excessive ROS could attack the mitochondrial membrane, leading to impaired mitochondrial structure and function, whereas Ex-4 preconditioning accelerated the clearance of ROS by improving the antioxidant defense ability of ADMSCs. So this ROS-scavenging action could also be involved in the protective effect of Ex-4 on mitochondrial function.

Thus, on one hand, we have provided evidence that H_2O_2 causes ADMSCs death via mitochondrial apoptosis pathways, which is largely in agreement with several previous studies [52], indicating a potential therapeutic target that may promote the survival of engrafted ADMSCs in regenerative medicine. Yet, there are other apoptotic routes, including caspase-8-mediated death-receptor pathways [53] and

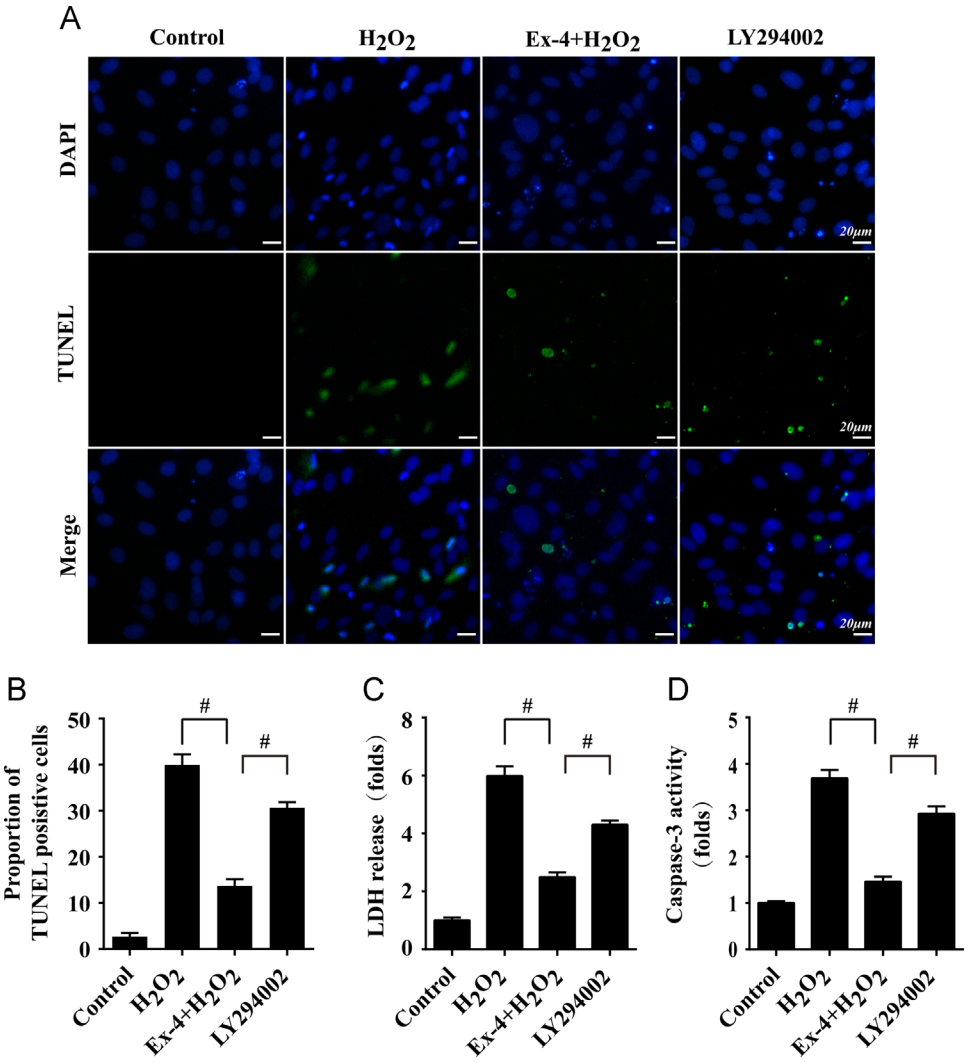

Fig. 6. The PI3K/Akt pathway was involved in the beneficial action of Ex-4. (A and B) Percentage of TUNEL-positive cells with and without LY294002 application. Apoptotic nuclei were identified as TUNEL positive (green). (C) LDH release assay. (D) Caspase-3 activity was measured after the PI3K/Akt pathway was inhibited by LY294002. Cell nuclei are blue. Bar, 50 μm; $^{\#}p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

caspase-12-involved endoplasmic reticulum stress pathways [54], that are likely to participate in the cell death along with the progression of oxidative stress injury. However, whether these death signals have a role in ADMSCs apoptosis and which one signal is the main mechanism of early cell apoptosis after transplantation in vivo remain unclear. More insight into the interaction of these apoptosis pathways on ADMSCs under oxidative injury should be obtained. On the other hand, we proved that Ex-4 plays a pivotal role in protecting ADMSCs from H_2O_2-induced apoptosis by scavenging ROS and increasing antiapoptotic proteins that protect mitochondria against oxidative injury, showing that Ex-4 could be used as an adjuvant to strengthen the intrinsic repair system of ADMSCs against apoptosis under oxidative conditions. Recent studies have identified exendin-4 as a novel antidiabetic agent, which was primarily designed to improve glycemic control, and researchers have lately further found that Ex-4 plays a key role in cardioprotection, including reduction in infarction size, improvement in LVEF (left ventricular ejection fraction), reversion of cardiac remodeling [55], and inhibition of cardiomyocyte apoptosis [11]. In particular, our study broadened the potential application of Ex-4 by demonstrating the beneficial effects of Ex-4 on ADMSCs apoptosis. These data altogether hinted that Ex-4 could be a new therapeutic tool for the injured heart by improving cardiac function and enhancing the survival ability of exogenous engrafted cells in the hostile microenvironment.

In our previous studies, we have demonstrated that Akt may be the molecular signal for Ex-4-induced recruitment of antiapoptotic proteins and ADMSCs survival [14,15]. However, the detailed downstream signal of Akt remains unclear. It has been shown that activated PI3K/Akt could improve the expression of Sfrp2 [34], which was identified as the new major factor mediating MSC survival under hypoxic conditions through inactivation of caspase networks [35]. Thereby, we speculated whether activated Akt induced by Ex-4 could stimulate Sfrp2 expression in ADMSCs and whether the activated Sfrp2 was related to the balance of anti- and proapoptotic proteins and the stabilization of mitochondrial function. In the present study, we found that Ex-4 increased the expression of p-Akt as well as Sfrp2, and Ex-4-induced upregulation of Sfrp2 was PI3K/Akt dependent, because inhibition of PI3K/Akt with LY294002 abolished Sfrp2 expression in ADMSCs. Moreover, knockdown of Sfrp2 expression with siRNA abrogated the beneficial effects of Ex-4 on mitochondrial function under oxidative stress with evidence showing reduced $\Delta\Psi m$ and more Cyt *c* release. Furthermore, siRNA against Sfrp2 resulted in higher contents of the proapoptotic proteins Bax and caspase-9/3 as well as lower levels of the antiapoptotic proteins Bcl-2 and c-IAP1/2. These data indicated that Sfrp2 protected mitochondrial function by regulating the balance of anti- and proapoptotic proteins, which was in line with previous reports that Sfrp2 was a principal

prosurvival signal in several kinds of cells [56,57]. Meanwhile, inhibition of Akt caused more apoptosis in cells and higher caspase-3 activity compared with the Ex-4 group, indicating that PI3K/Akt was also necessary for the antiapoptotic action of Ex-4. Altogether, we established the role of the PI3K/Akt–Sfrp2 signaling pathways in Ex-4's antiapoptotic effects on ADMSCs. Furthermore, the detailed mechanism by which Sfrp2 regulates the levels of apoptosis-related proteins and inhibits cellular apoptosis may be associated with the stabilization or degradation of β-catenin. Recent studies found that Sfrp2 activated by Akt in cardiomyocytes could increase cytoplasmic β-catenin translocation into the nucleus, where it acts as a transcription factor promoting the expression of survival genes such as Bircb [16]. In ADMSCs, higher Sfrp2 induced by Akt also resulted in more β-catenin that contributed to cell survival [34], but the detailed genetic changes underlying the PI3K/Akt–Sfrp2–β-catenin pathways in ADMSCs require further investigation.

In summary, the present findings reveal that mitochondria play an important role in the transmission and amplification of apoptosis signals, indicating that protection of mitochondrial function in situations of oxidative stress may be an important therapeutic target for increasing ADMSCs survival. Furthermore, Ex-4 intervention is a useful tool for reduction of ADMSCs apoptosis under oxidative stress, and the PI3K/Akt–Sfrp2 pathway is the downstream prosurvival signal that inactivates the caspase-9-mediated mitochondrial death pathway and terminal caspase networks, and therefore, these data offer an attractive way to maximize the effectiveness of ADMSCs-based therapy after MI.

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