Original Article

ROS activates JNK-mediated autophagy to counteract apoptosis in mouse mesenchymal stem cells *in vitro*

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Aim: Transplantation of mesenchymal stem cells (MSCs) for the treatment of diabetic erectile dysfunction (ED) is hampered by apoptosis of the transplanted cells. In diabetic ED, there is increased oxidative stress and decreased NO in the corpora cavernosa, and reactive oxygen species (ROS) induce apoptosis of the transplanted cells. In this study we examined whether and how autophagy was involved in ROS-induced apoptosis of MSCs.

Methods: Mouse C3H10 MSCs were treated with H₂O₂ to simulate the high oxidative condition in diabetic ED. Cell viability was measured using MTT assay. Apoptosis was analyzed by flow cytometry. Apoptosis- and autophagy-related proteins were detected with Western blot assays. Intracellular autophagosome accumulation was studied using transmission electron microscopy.

Results: Treatment of MSCs with H₂O₂ (50–400 μmol/L) inhibited the cell viability in concentration- and time-dependent manners. Furthermore, H₂O₂ (300 μmol/L) induced apoptosis, as well as activated autophagy in MSCs. Pretreatment with lysosome inhibitor chloroquine (10 μmol/L) or PI3K inhibitor 3-methyladenine (5 mmol/L) significantly enhanced H₂O₂-induced cell death. Pretreatment with JNK inhibitor SP600125 (10 μmol/L) abrogated H₂O₂-induced accumulation of LC3-II, and attenuated H₂O₂-induced reduction of Bcl-2 levels in MSCs.

Conclusion: ROS induce autophagy to counteract apoptosis in MSCs by activation of JNK. Thus, augmentation of autophagy may reduce apoptosis, prolonging MSC survival and improving MSC-based therapeutic efficacy for diabetic ED.

Keywords: mesenchymal stem cell; ROS; autophagy; apoptosis; chloroquine; 3-methyladenine; SP600125; diabetes mellitus; erectile dysfunction

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Introduction

Diabetic patients suffer from a higher prevalence of erectile dysfunction (ED) and are less responsive to oral anti-ED drugs than non-diabetic individuals, which seriously affects the quality of life of diabetic patients^[1–4]. Phosphodiesterase inhibitor 5 (PDEI5) (*eg*, sildenafil, vardenafil and tadalafil) represents the first-line therapeutic strategy for ED, but there are side effects of these drugs (*eg*, headache, flushing and diarrhea). Disappointing effects in diabetes patients compared with the general population have been reported^[5–7]. Because stem cells can directionally differentiate into corpus cavernosum vascular endothelial cells and smooth muscle cells under a specific microenvironment, the transplantation of stem cells

for treating diabetic ED was proposed as a potential therapeutic approach^[8–10]. Mesenchymal stem cells (MSCs) have been tested in transplantation therapy for diabetic ED^[11–13]. Although stem cell transplantation can improve erectile function in animal studies, the effect last only 7–28 d before gradually diminishing^[14–16]. The main mechanism of these limited effects is assumed to be the apoptotic death of the transplanted stem cells. Thus, strategies that suppress apoptosis would increase stem cell survival and improve therapeutic efficacy.

Autophagy is an evolutionarily conserved catabolic process involved in the degradation and recycling of long-lived proteins and the removal of exhausted and unwanted cellular components, which is important for cell survival under various stress conditions such as nutrient starvation, reactive oxygen species (ROS) accumulation and infection. Consistent with its vital role in cell survival, proliferation and differentiation, autophagy is important in retaining the survival and

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function of stem cells^[17]. Thus, modulating autophagy may be used to prolong stem cell survival and improve stem cell-based therapy.

Recent studies demonstrate that there is increased oxidative stress and decreased production of nitric oxide in the corpora cavernosa^[18, 19]. Reactive oxygen species (ROS) are toxic to transplanted stem cells, and the induction of apoptosis underlies the major mechanism of ROS-induced cytotoxicity^[20–23]. Meanwhile, ROS can also activate autophagy in certain conditions^[24–26]. The relationship between autophagy and apoptosis under oxidative conditions is complex^[27]. Whether autophagy is activated in transplanted MSCs and the relationship between autophagy and apoptosis remain unclear.

In this report, we demonstrate that ROS kills MSCs through apoptosis. Meanwhile, it induces autophagy through JNK-mediated Bcl-2 degradation. While autophagy counteracts apoptosis, the latter is overwhelming. Therefore, our results suggest that the augmentation of autophagy could be exploited to suppress apoptosis, which may prolong transplanted MSC survival and improve MSC-based therapy for diabetic ED.

Materials and methods

Materials

Antibodies for Bcl-2 (#9941), phospho-JNK (#4668), JNK1 (#3708) and cleaved-caspase 3 (#9664) were purchased from Cell Signaling Technology (Cell Signaling Technology, Danvers, MA, USA). Anti-PARP and p62 (610497) were purchased from BD Biosciences (New York, USA). Antibodies for LC3B (L7543) and chloroquine (CQ, 50-63-5) were purchased from Sigma Aldrich (St Louis, MO, USA). The antibody against β -actin was purchased from Zoonbio Biotechnology (Nanjing, China). All secondary antibodies were purchased from Abgent (San Diego, CA, USA). The JNK inhibitor SP600125 (s1460) and pan-caspase inhibitor z-VAD were purchased from Selleckchem (Houston, TX, USA). The 3-methyladenine (3MA) (sc-205596) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dulbecco's modified Eagle's medium/F12 (DMEM/F12) and trypsin were purchased from HyClone (Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Gibco (Thermo Fisher Scientific, MA, USA).

Cell culture

The mice mesenchymal stem cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM/F12 containing 10% FBS, 1 mmol/L-glutamine, 1×10^5 units/L penicillin and 100 g/L streptomycin and maintained at 37°C with 5% CO₂ in a humidified atmosphere.

Measurement of cell viability

Cell viability was assessed by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT; Beyotime, Haimen, China) assay. Briefly, cells were seeded into 96-well plates at 1×10^4 cells/cm² and cultured for 24 h. The cells were then pre-treated with the indicated inhibitors for 30 min by exposure to

H₂O₂ for another 12, 24, or 48 h. After incubation, the medium was aspirated, and the cells were incubated with fresh medium containing 5 g/L MTT. After 4 h, the medium was removed, and the blue formazan crystals were dissolved in 150 μ L dimethyl sulfoxide (DMSO). Absorbance (λ /nm=570) was measured using a Tecan Infinite F200/M200 type multifunction microplate reader (Tecan, Männedorf, Switzerland). The viability rate of the cells=(the OD values of treated groups/the OD values of control group) $\times 100\%$.

Western blot analysis

To obtain the total protein lysates, treated cells were lysed in cold RIPA lysis buffer (Beyotime, Haimen, China) containing 1 mmol/L phenylmethane-sulfonyl fluoride (PMSF; Beyotime, Haimen, China), 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail (Biotool, Houston, TX, USA) and centrifuged at 12 000 $\times g$ for 15 min at 4°C to remove debris. Protein concentrations were estimated using the enhanced bicinchoninic acid (BCA) protein assay kit (Beyotime, Haimen, China), and the protein extracts were heat denatured in SDS-PAGE sample loading buffer (Beyotime, Haimen, China). Equal amounts of protein (40 μ g/lane) from each sample were separated by 10%–12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the criterion system at a constant voltage of 90 V. The proteins were subsequently transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany). After blocking with 5% non-fat dried milk for 2 h, the membrane was incubated with the primary antibodies overnight at 4°C. Then, the immunoreactive bands were visualized by enhanced chemiluminescence using HRP-conjugated IgG secondary antibodies.

Flow cytometric (FCM) analysis of apoptosis

After treatment, the cells were trypsinized, washed with PBS and suspended in 195 μ L of Annexin V-FITC binding buffer containing 5 μ L Annexin V-FITC and 10 μ L propidium iodide (PI; Beyotime, Haimen, China). After incubation for 10–20 min at room temperature in the dark, the cells were subjected to a FCM assay. FCM was performed using a FACSCanto 6-color flow cytometer (BD Biosciences, San Jose, CA, USA).

Transmission electron microscopy (TEM)

For the TEM analysis, treated cells were trypsinized, rinsed twice with warm PBS (37°C) and then fixed for 1 h in 2.5% glutaraldehyde in 0.1 mol cacodylate buffer with 1% sucrose. After washing with PBS, the cells were postfixed in 1% osmium tetroxide (OsO₄) and embedded in Epon; 0.1 mmol/L thin sections were stained with uranyl acetate/lead citrate and viewed using a Hitachi-7500 TEM (Tokyo, Japan).

Statistical analysis

All data are expressed as the mean $\pm$ SD. The SPSS19.0 software package was used to perform all statistical analysis. Statistical comparisons were performed by one-way ANOVA. In all analysis, $P < 0.05$ was considered statistically significant.

Results

H₂O₂ reduced cell viability in a dose- and time-dependent manner

We used H₂O₂ to simulate a high oxidative condition. MSCs were exposed to various concentrations of H₂O₂ (0, 50, 100, 200, 300, and 400 µmol/L) for 12, 24, and 48 h, and cell viability was measured by the MTT assay. H₂O₂ reduced cell viability in a dose- and time-dependent manner (Figure 1) with an IC₅₀ of 325.9352±35.39494 µmol/L at 24 h. Thus, 300 µmol/L of H₂O₂ was chosen for subsequent experiments.

ROS induce apoptosis in MSCs

Consistent with reported studies^[20-23], we determined that H₂O₂ induced apoptosis in MSCs, which was shown by the activation of caspase 3 and cleavage of poly-ADP-ribose polymerase (PARP) (Figure 2), positive Annexin V staining (Figure 3B) and suppression of cytotoxicity by the pan-caspase inhibitor z-VAD (Figure 3A, 3B).

Autophagy inhibition enhanced H₂O₂-induced apoptosis in MSCs

To understand the relationship between autophagy and apoptosis in high-ROS conditions, we examined the effects of autophagy inhibition on H₂O₂-induced apoptosis. The phosphatidylinositol 3-kinase (PI3K) inhibitor 3-MA and the lysosome inhibitor CQ, which inhibit different steps of the autophagy process, were used to pretreat the cells before H₂O₂

exposure. As shown in Figure 3A, cell viability was further decreased when autophagy was inhibited by 3-MA or CQ, suggesting that autophagy is cytoprotective under this condition. Consistently, the inhibition of autophagy enhanced apoptosis, as shown by increased Annexin V staining, elevated cleavage of caspase 3 and PARP in CQ pre-treated cells (Figure 3B, 3C).

H₂O₂ induced autophagy in MSCs

To investigate whether H₂O₂ was able to induce autophagy in MSCs, we used TEM to detect the autophagosome, the autophagic vacuoles that are the morphological hallmark of autophagy^[28]. As shown in Figure 4A, autophagic vacuoles containing cellular material or membranous structures in H₂O₂-treated cells were detected. The conversion of LC3-I to LC3-II and the degradation of p62, markers of autophagy^[28, 29], were also detected by Western blot (Figure 4B). Additionally, autophagy flux was detected through co-treatment with H₂O₂ and CQ, a lysosome inhibitor that inhibits LC3-II turnover. In the experiment, while either H₂O₂ or CQ alone caused moderate increases in LC3-II, the exposure of cells to both H₂O₂ and CQ further increased LC3-II expression (Figure 4C). Together, these results confirmed that autophagy was induced by H₂O₂ treatment.

H₂O₂ induced autophagy was mediated by JNK activation.

Previously, it was demonstrated that autophagy can be induced through the JNK-mediated phosphorylation or degradation of Bcl-2 and Bcl-xL, which attenuates the inhibition of Bcl-2 and Bcl-xL on Beclin-1^[30, 31]. To investigate whether JNK is involved in autophagy induced by H₂O₂, cells were treated for different time periods, and the phosphorylation of JNK, the activated form of JNK, was detected by Western blot. As shown in Figure 5A, JNK was significantly activated by H₂O₂. Meanwhile, the expression of Bcl-2 was decreased in a time-dependent manner.

To further determine the role of JNK in autophagy induced by H₂O₂, the JNK inhibitor SP600125 was used to inhibit JNK. SP600125 abrogated the effects of H₂O₂ on the accumulation of LC3-II, which was associated with inhibiting the decrease in Bcl-2 (Figure 5B and 5C). These results suggest that H₂O₂-induced autophagy involves JNK-mediated Bcl-2 suppression.

Discussion

ROS-mediated apoptosis limits the efficacy of stem cell-based therapy for diabetic ED. In this report, we investigated the role of autophagy in ROS-induced apoptosis in MSCs and found that while H₂O₂ killed MSCs through apoptosis, autophagy was also effectively activated by ROS. Blocking autophagy significantly increased apoptotic cytotoxicity, suggesting that ROS-activated autophagy was cytoprotective against apoptosis. Further, we found that H₂O₂ activated JNK and suppressed Bcl-2. These results demonstrate that while ROS kills MSCs through apoptosis, it also induces cytoprotective autophagy. It appears that the apoptosis is overwhelming, and autophagy is unable to override apoptosis. Thus, the

Figure 1. Assessment of the effects of H₂O₂ on MSC viability. Cells were treated with various concentrations of H₂O₂ for 12, 24, and 48 h, and cell viability was measured by the MTT assay and the cell viability ratio (%) was calculated. Data from 3 independent experiments are represented as the mean±SD. ^b*P*<0.05 vs control group (0 µmol/L).

Figure 2. H₂O₂ induces apoptosis in MSCs. The cells were treated with H₂O₂ (300 µmol/L) for the indicated time periods. The indicated proteins were detected by Western blot, and β-actin was detected as an input control.

Figure 3. Blockage of autophagy strengthens the cytotoxicity of H₂O₂. (A) Cells were pretreated with 3MA (5 mmol/L), CQ (10 μmol/L) or z-VAD (20 μmol/L) for 30 min and then with H₂O₂ (300 μmol/L) for 24 h. Cell viability was measured by MTT assay. (B) Cells were pretreated with CQ (10 μmol/L) or z-VAD (20 μmol/L) for 30 min and then with H₂O₂ (300 μmol/L) for 8 h. Cell apoptosis was measured by flow cytometry (FCM). Annexin V⁺/PI⁻ represents the early apoptotic stage, whereas Annexin V⁺/PI⁺ represents apoptotic cells in the terminal stage. Necrotic cells display Annexin V⁻/PI⁺. The percentages of early and terminal stage apoptotic cells and necrotic cells were calculated and are shown in the histogram. (C) MSCs were pretreated with CQ (10 μmol/L) or z-VAD (20 μmol/L) for 30 min and then treated with H₂O₂ (300 μmol/L) for 8 h. The indicated proteins were detected by Western blot, with β-actin detected as an internal control. Mean±SD. *n*=3. ^a*P*<0.05 vs control group; ^b*P*<0.05 vs the H₂O₂ group; ^c*P*<0.05 vs the (H₂O₂+CQ) group.

Figure 4. H₂O₂ induces autophagy in MSCs. (A) Cells were treated with or without H₂O₂ (300 μmol/L) for 4 h, and autophagosomes were detected by TEM. Black arrows indicate autophagosomes including residual digested organelles. (B) Cells were treated with H₂O₂ (300 μmol/L) for the indicated times. The indicated proteins were detected by Western blot, and β-actin was detected as a loading control. (C) The cells were pretreated with CQ (10 μmol/L) for 30 min and then treated with H₂O₂ (300 μmol/L) for another 2 h. LC3 was detected by Western blot, and β-actin was detected as a loading control.

Figure 5. JNK activation is required for H₂O₂-induced autophagy. (A) Cells were exposed to H₂O₂ (300 μmol/L) for different durations. The indicated proteins were detected by Western blot, and β-actin was detected as a loading control. (B and C) Cells were pretreated with SP600125 (10 μmol/L) for 30 min and then treated with H₂O₂ (300 μmol/L) for an additional 2 h (for LC3) or 1 h (for Bcl-2). The indicated proteins were detected by Western blot, and β-actin was detected as a loading control.

augmentation of autophagy would reduce apoptosis and thus prolong transplanted MSC survival, which might be applicable in MSC-based therapy for diabetic ED.

The high blood glucose in diabetes mellitus puts the body in a state of high oxidative stress, with damage to the corpora cavernosa nerves and endothelial cells, as well as inactivation of neuronal and endothelial nitric oxide synthase, leading to decreased production of nitric oxide and diabetes mellitus erectile dysfunction^[18, 19]. In the rat model of diabetic ED caused by streptozotocin, the products of oxidative stress, such as malonaldehyde, were accumulated, while antioxidant substances (eg, SOD and glutathione) were decreased, resulting in increased ROS levels in the corpus cavernosum^[32, 33]. Recent investigations have demonstrated that ROS affect the self-renewal and differentiation of bone marrow mesenchymal stem cells, accelerate aging in stem cells and endothelial progenitor cells, increase apoptosis and reduce the self-healing of damaged blood vessels^[34, 35]. Consistently with the reports that ROS can induce apoptosis, we show that ROS can kill MSCs through the activation of apoptosis, which is shown by caspase 3 activation, PARP cleavage, increased Annexin V staining and suppression of ROS cytotoxicity by z-VAD. Thus, ROS-induced apoptosis plays an important role in stem cell viability and function and the pathogenesis of diabetic ED, and the manipulation of ROS-induced apoptosis may be

usable to treat diabetic ED.

In addition to apoptosis, ROS also induces autophagy under certain circumstances. As a cellular catabolic process, autophagy plays an important role in stem cell differentiation and self-renewal^[17, 36, 37]. In our study, we clearly demonstrate that H₂O₂ induces autophagy in MSCs, including increased LC3-II expression, decreased p62 expression, and autophagic flux. Autophagy can mediate cell survival or death, depending on the cell type, environment and stimulation^[31, 38, 39]. The role of ROS-induced autophagy in MSCs is unclear. In our study, ROS-induced autophagy is cytoprotective, as the suppression of autophagy increases the apoptosis and cytotoxicity induced by ROS, which is consistent with a recent report on hepatocytes^[40].

MAPKs such as JNK are involved in the induction of autophagy through Bcl-2 phosphorylation and disruption of the Bcl-2/Beclin1 complex^[30]. We found that ROS potently activated JNK while decreasing Bcl-2 expression in MSCs. The pharmacological inhibition of JNK significantly suppressed autophagy, implying the important role of JNK in ROS-induced autophagy in MSCs. Further, JNK inhibitor attenuated Bcl-2 reduction. While previous studies have shown that Bcl-2 phosphorylation can disrupt the Bcl-2/Beclin1 complex for autophagy activation^[30], we recently found that the JNK-mediated degradation of Bcl-2 also leads to the induction of autophagy^[31]. Thus, it is possible to conclude that JNK-mediated Bcl-2 degradation results in Beclin1-mediated autophagy activation in ROS-treated MSCs.

In summary, we demonstrate that while ROS kills MSCs through apoptosis, it also induces cytoprotective autophagy. Because the apoptosis is overwhelming, autophagy is unable to override apoptosis. Therefore, the augmentation of autophagy could reduce apoptosis and prolong transplanted MSC survival, improving MSC-based therapy for diabetic ED. Further *in vivo* studies are needed to test this hypothesis.

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Author contribution

Xin GOU, Wei-yang HE and Yong LIN designed the research; Guan-yu LIU and Xiao-xue JIANG performed the experiments; Guan-yu LIU, Xiao-xue JIANG, You-lin KUANG and Ke REN analyzed the data; and Yong LIN, Guan-yu LIU and Xin ZHU wrote the paper.

Abbreviations

3-MA, 3-methyladenine; Bcl-2, B-cell lymphoma 2; CQ, chloroquine; DMSO, dimethyl sulfoxide; ED, erectile dysfunction; FCM, flow cytometric; JNK, c-Jun N-terminal kinase; LC3, microtubule associated protein 1 light chain 3; MAPKs, mito-

gen-activated protein kinases; MSCs, mesenchymal stem cells; MTT, methyl thiazolyl tetrazolium; OD value, optical density value; PARP, poly(ADP-ribose) polymerase; PDE15, phosphodiesterase type-5 inhibitors; PMSF, phenylmethylsulphonyl fluoride; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; TEM, transmission electron microscopy.

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