

Inhibition of Store-Operated Calcium Entry Protects Endothelial Progenitor Cells from H₂O₂-Induced Apoptosis

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Abstract

Store-operated calcium entry (SOCE), a major mode of extracellular calcium entry, plays roles in a variety of cell activities. Accumulating evidence indicates that the intracellular calcium ion concentration and calcium signaling are critical for the responses induced by oxidative stress. The present study was designed to investigate the potential effect of SOCE inhibition on H₂O₂-induced apoptosis in endothelial progenitor cells (EPCs), which are the predominant cells involved in endothelial repair. The results showed that H₂O₂-induced EPC apoptosis was reversed by SOCE inhibition induced either using the SOCE antagonist ML-9 or via silencing of stromal interaction molecule 1 (STIM1), a component of SOCE. Furthermore, SOCE inhibition repressed the increases in intracellular reactive oxygen species (ROS) levels and endoplasmic reticulum (ER) stress and ameliorated the mitochondrial dysfunction caused by H₂O₂. Our findings provide evidence that SOCE inhibition exerts a protective effect on EPCs in response to oxidative stress induced by H₂O₂ and may serve as a potential therapeutic strategy against vascular endothelial injury.

Key Words: Endothelial progenitor cells, Oxidative stress, SOCE, STIM 1, ML-9

INTRODUCTION

Endothelial dysfunction plays a central role in the development and progression of vascular diseases. Endothelial progenitor cells (EPCs), which are mobilized from bone marrow to the vascular endothelium, are vital for endothelial repair, functioning directly through differentiation and indirectly through paracrine effects. Oxidative stress caused by free radicals, oxygen anions and peroxides is closely associated with the initiation, development and progression of vascular diseases (Heitzer *et al.*, 2001; Yoshii *et al.*, 2006; Schleicher and Friess, 2007; Cachofeiro *et al.*, 2008; Battelli *et al.*, 2014) and has direct cytotoxic effects on the endothelium (Griendling and FitzGerald, 2003; Harrison *et al.*, 2003) and EPCs (Hung *et al.*, 2003; Urbich *et al.*, 2005; Thum *et al.*, 2006; Thum *et al.*, 2007; Watson *et al.*, 2008). However, the specific mechanism of these effects is not clear, and the roles of calcium and calcium signaling in this mechanism are undefined.

Calcium is an important ion that regulates a variety of activities, including muscle contraction, skeleton formation, and cell proliferation, migration and apoptosis. Store-operated calcium channels (SOCs; also known as SOCCs) mediate store-operated calcium entry (SOCE) and are expressed ubiquitously

in non-excitabile cells, such as EPCs (Lewis, 2007). Previous studies have demonstrated that SOCE in embryonic fibroblasts is critical for the oxidative stress response (Henke *et al.*, 2012). Numerous studies have suggested that oxidative stress enhances the inward flow of calcium, leading to intracellular calcium overload (Caron *et al.*, 2007) and ultimately, cell apoptosis (Henke *et al.*, 2013). Therefore, we hypothesized that SOCE may play a significant role in oxidative stress-induced apoptosis in EPCs. Inhibition of SOCE may protect EPCs from oxidative stress-induced apoptosis, thus making SOCE a potential target for the treatment of various vascular diseases.

Stromal interaction molecule (STIM) proteins are localized to the endoplasmic reticulum (ER) and act as sensors of ER calcium. These proteins play a direct role in the ER-plasma membrane (PM) junction (Rebecchi and Pentylala, 2000; Liou *et al.*, 2005). They regulate extracellular calcium via SOCE or capacitative calcium entry (Roos *et al.*, 2005). Studies by our group have indicated that both shRNA (shSTIM1) and ML-9, an SOCE antagonist, protect EPCs from injuries induced by oxidative stress. Further work has suggested that this protection may involve interference with multiple factors associated with intracellular reactive oxygen species (ROS), ER stress

Open Access <http://dx.doi.org/10.4062/biomolther.2015.130>

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Received Aug 17, 2015 Revised Nov 9, 2015 Accepted Nov 17, 2015
 Published Online Jul 1, 2016

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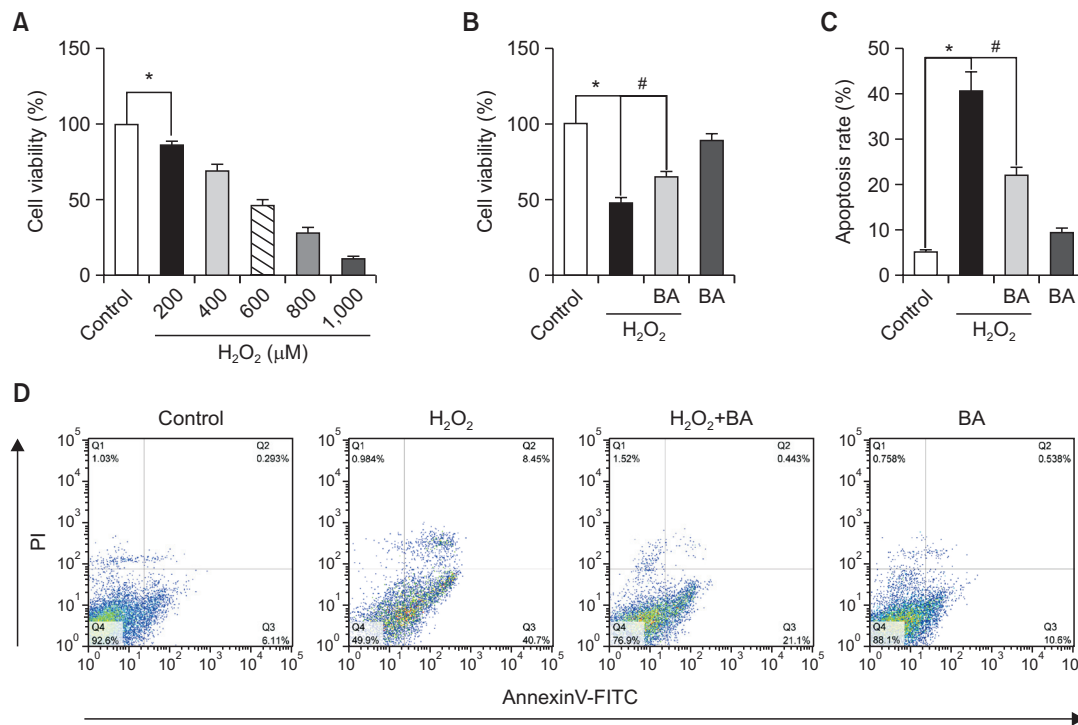


Fig. 1. H₂O₂-induced cytotoxicity and apoptotic cell death involve disruption of intracellular calcium homeostasis. After treatment with different concentrations of H₂O₂ for 6 h, cell viability was measured using a CCK-8 kit (A). After pretreatment with BAPTA-AM (BA), cells were treated with H₂O₂ for 6 h, and cell viability was measured (B). EPCs were pretreated with BAPTA-AM (BA) before exposure to H₂O₂ for 6 h. The cells were then stained with Annexin V-FITC and propidium iodide (PI) and analyzed via flow cytometry (D). Quantitative data for Annexin V-FITC/PI staining is presented (C). **p*<0.05; #*p*<0.05.

and mitochondrial dysfunction.

MATERIALS AND METHODS

Cell culture and characterization of EPCs

Male Sprague-Dawley rats were sacrificed by injection of an overdose of sodium pentobarbital (100 mg/kg) into the peritoneal cavity (Zhao *et al.*, 2008), according to the standards of the American Veterinary Medical Association Panel on Euthanasia. Bone marrow-derived EPCs (BM-EPCs) were isolated from the femurs and tibias of rats through density-gradient centrifugation (Sigma-aldrich, St. Louis, USA) according to established methods. Isolated EPCs were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, USA) at 37°C under 5% CO₂ and 100% humidity. EPCs were cultured for 7 days before being utilized in experiments. To confirm the identities of EPCs, cells were incubated with acLDL-Dil (10 mg/ml) for 6 h and then fixed with 4% paraformaldehyde for 15 min, incubated with fluorescein isothiocyanate-labeled lectin (UEA-1; 10 mg/ml) for 1 h and examined under a laser confocal scanning microscope (Leica, Wetzlar, Germany) (Kuang *et al.*, 2012). Cells that were double positive for acLDL-Dil and UEA-1 were identified as EPCs. Approximately 85% of cells were positive for both markers.

ShRNA transfection

STIM1 expression was silenced using a lentiviral vector carrying stromal interaction molecule 1 (STIM1) shRNA con-

structed by GenePharma (Shanghai, China). EPCs were cultured in 6-well plates 2 days before transfection. Cells were infected with the lentiviral vector at a multiplicity of infection of 100 for 48 h. The STIM1 shRNA sequence was GCGACTTCT-GAAGAGTCTACC. The negative control shRNA sequence was TTCTCCGAACGTGTCACGT. The efficiency of shRNA transfection was confirmed by Western blot analysis.

Cell viability assay

A cell viability assay was performed using a Cell Counting Kit-8 (CCK-8) (Beyotime, Jiangsu, China) in accordance with the manufacturer's protocol. After 7 days of culturing, EPCs were trypsinized (HyClone, Utah, USA) and seeded into 96-well plates (approximately 5,000 cells/well). Cell viability was quantified by measuring absorbance at 450 nm with a microplate reader (Molecular Devices, Sunnyvale, USA) after cells were treated with processing factors and incubated with CCK-8. The results were expressed as a percentage of the control group (Hou *et al.*, 2007; Zhang and Sun, 2010).

Apoptosis assay

EPC apoptosis was evaluated with a FITC-Annexin V Apoptosis Detection Kit (Neo Bioscience, Beijing, China). After 7 days of culturing, EPCs were treated with processing factors and then trypsinized and treated with Annexin V-FITC and propidium iodide (PI). The treated cells were finally counted via flow cytometry (Beckman coulter, Indianapolis, USA) following the manufacturer's protocol. The results were expressed as the percentages of cells in the second quadrant

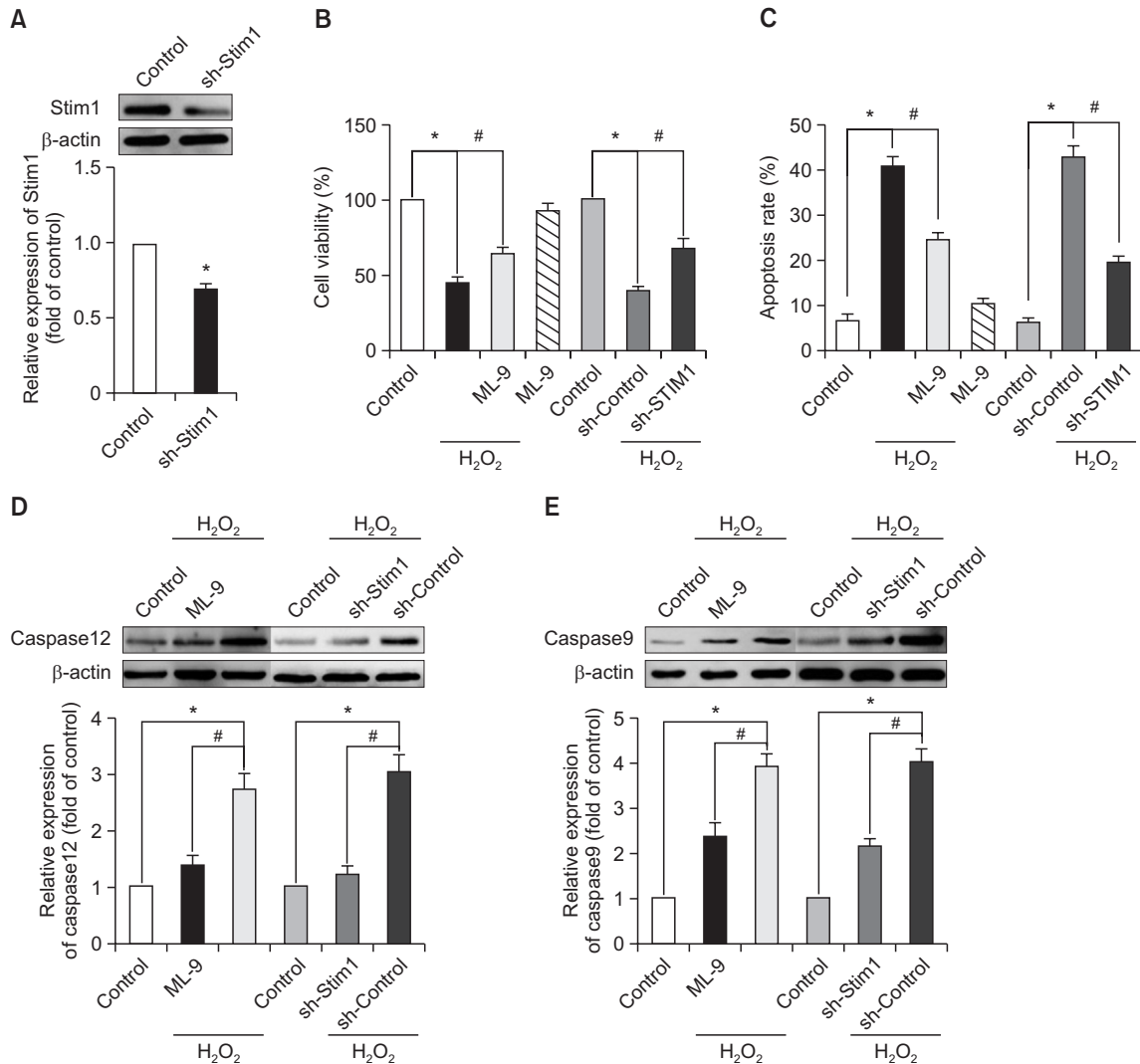


Fig. 2. Inhibition of SOCE attenuates the detrimental effects of H₂O₂ on EPCs. Representative immunoblots (top) and quantification (bottom) of STIM1 expression 48 h after transfection (A). EPCs that had been cultured for 7 days were pretreated with ML-9 for 20 min or with STIM1 shRNA before the addition of H₂O₂ for 6 h. Cell viability (B) and apoptosis (C) were then measured. The relative expression levels of caspase 12 (D) and caspase 9 (E) were measured by Western blot analysis. **p*<0.05; #*p*<0.05.

(early apoptosis) and third quadrant (late apoptosis).

Calcium imaging

Intracellular calcium concentration was measured with the Fluo-3AM calcium indicator. EPCs were pretreated with 50 μM ML-9 or shSTIM1, incubated with Fluo-3AM for 30 min in the dark, washed 3 times with phosphate-buffered saline (PBS), incubated in medium for 30 min, washed with PBS three times and placed in a calcium-containing or calcium-free solution. The cells were then observed under a laser scanning confocal microscope at an excitation wavelength of 488 nm and emission wavelengths alternating between 525 nm and 530 nm. The results were analyzed with specific software (Li *et al.*, 2009).

Detection of ROS in EPCs

To detect intracellular ROS in EPCs, we used a Reactive Oxygen Species Assay Kit (Beyotime), which detects changes

in intracellular ROS using the DCFH-DA fluorescent probe. EPCs were treated with processing factors and then incubated with DCFH-DA and observed under a laser scanning confocal microscope at a 488 nm excitation wavelength and a 525 nm emission wavelength. The results were evaluated based on the intensity of the intracellular fluorescence (Jia *et al.*, 2006).

Measurement of the mitochondrial membrane potential (MMP)

The MMP was measured using the fluorescent dye rhodamine123 (Rh123) (Beyotime). Rh123 rapidly passes through the PM and is sequestered by active mitochondria within a few minutes. In our experiments, EPCs were cultured with 2 μM Rh123 for 30 min at 37°C in the dark and then washed with PBS. The results were estimated by determining the gray values of the cells.

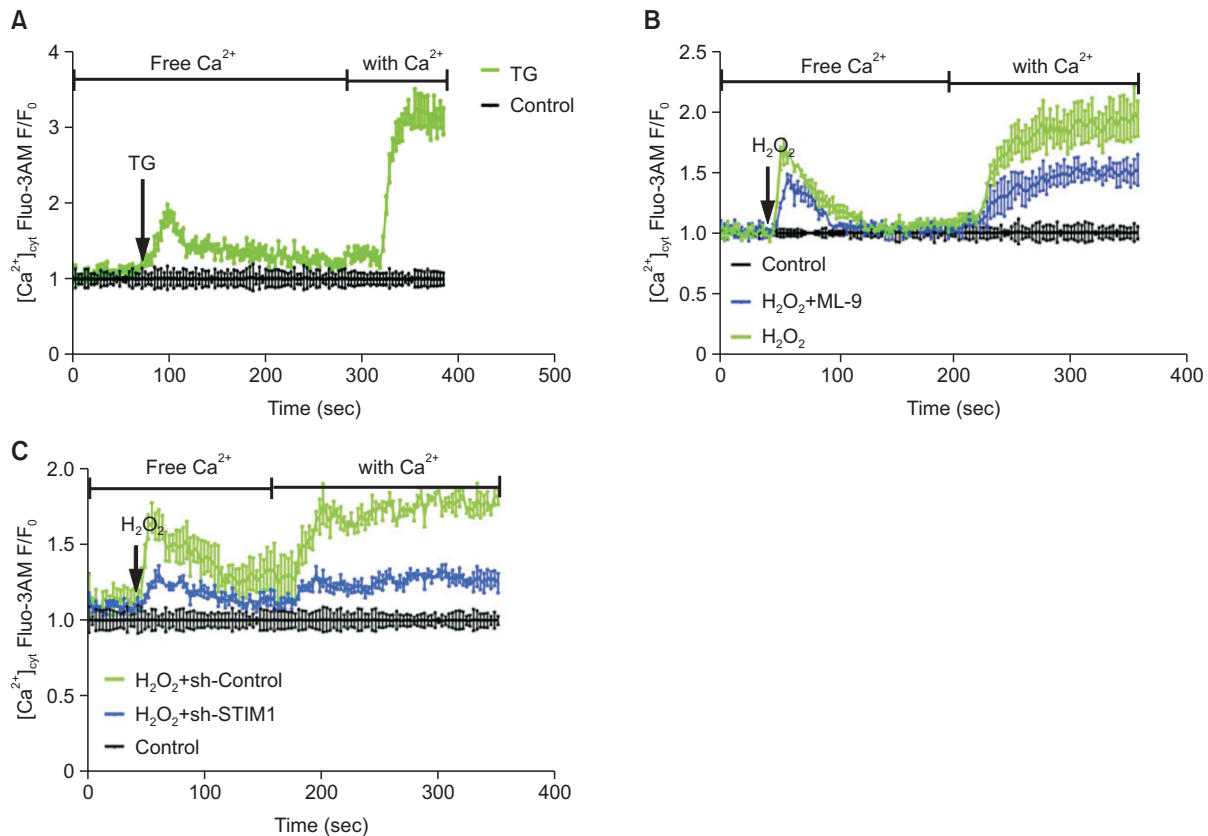


Fig. 3. Inhibition of SOCE restores intracellular calcium homeostasis. EPCs that had been cultured for 7 days were treated with thapsigargin (TG) (A) or pretreated with ML-9 for 20 min (B) or with STIM1 shRNA (C) before exposure to H₂O₂ in a calcium-free solution, followed by treatment with 2 mM CaCl₂. The intracellular calcium concentration was then measured using Fluo-3AM, a calcium indicator.

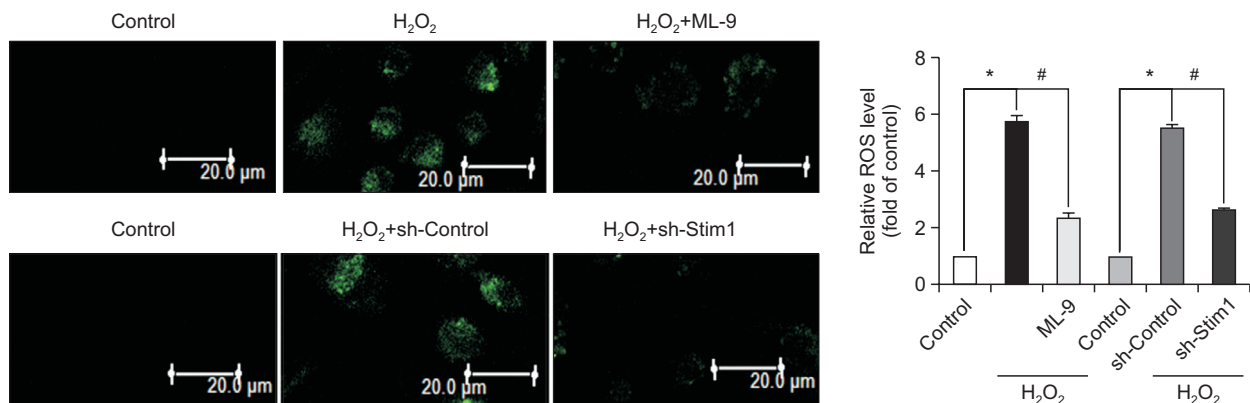


Fig. 4. Inhibition of SOCE prevents intracellular ROS accumulation. EPCs that had been cultured for 7 days were pretreated with ML-9 for 20 min or with STIM1 shRNA before exposure to H₂O₂ for 6 h, and intracellular ROS levels were evaluated using a fluorescence probe (DCFHDA). **p*<0.05; #*p*<0.05.

Western blot analysis

After the various treatments were performed, proteins were extracted from EPCs using a lysis buffer (RIPA) containing phenylmethanesulfonyl fluoride (PMSF). Protein concentrations were determined with a BCA protein assay kit. Equal amounts of protein (30 µg per lane) were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.5% Tween 20 and then incubated with specific primary antibodies. The membranes were subsequently incubated with appropriate secondary antibodies and then visualized with a luminescent image analyzer (GE, Connecticut, USA).

Statistical analysis

Statistical analyses were performed, and the data were expressed as the mean \pm standard deviation (SD) of five experiments. SPSS 16.0 was utilized for data analysis. The t-test was employed to compare two groups, and one-way analysis of variance (ANOVA) was used to compare multiple groups. A p -value < 0.05 was regarded as statistically significant.

RESULTS

H₂O₂-induced cytotoxicity and apoptotic cell death involve disruption of intracellular calcium homeostasis

EPCs (cultured for 7 days) were exposed to various concentrations (200, 400, 600, 800 and 1000 μ M) of H₂O₂ for 6 h, and cytotoxicity was determined with a CCK-8 kit. As shown in Fig. 1A, H₂O₂ induced cytotoxicity in a dose-dependent manner. When the H₂O₂ concentration was increased to 600 μ M, cell viability was approximately 50%. This concentration was used in the following experiments. H₂O₂-induced apoptosis was detected via flow cytometry. To assess whether intracellular calcium was involved in the H₂O₂-induced cytotoxicity and apoptosis of EPCs, we pretreated EPCs with BAPTA-AM (BA), an intracellular free calcium chelator (Fig. 1B-1D). The addition of BA (10 μ M) attenuated the H₂O₂-induced injury of EPCs, suggesting that H₂O₂-induced injury may be related to disruption of intracellular calcium homeostasis.

Inhibition of SOCE attenuates the detrimental effects of H₂O₂ on EPCs

To investigate the effects of SOCE inhibition, we utilized ML-9 (50 μ M), an antagonist of SOCE, and silenced the expression of STIM1 by shRNA (Fig. 2A). This method was used for SOCE in the following experiments. As hypothesized, the H₂O₂-induced decrease in EPC viability and increase in EPC apoptosis were reversed by the inhibition of SOCE (Fig. 2B, 2C). In addition, the H₂O₂-induced up-regulation of caspase 12, which is responsible for ER stress-induced apoptosis (Rasheva and Domingos, 2009), and caspase 9, which is closely related to the mitochondrial apoptotic pathway, was significantly attenuated by SOCE inhibition (Fig. 2D, 2E).

Inhibition of SOCE restores intracellular calcium homeostasis

Numerous data have demonstrated that calcium entry through SOCE channels is a common and ubiquitous mechanism of regulating calcium entry and that SOCE is the main mode of calcium entry into EPCs. Thus, we performed calcium imaging to determine whether H₂O₂ affects calcium signaling in EPCs through SOCE. H₂O₂ stimulation of EPCs in a calcium-free buffer led to an increase in cytosolic calcium due to calcium release from the ER (left peaks in Fig. 3B, 3C). The addition of calcium after cytosolic calcium level slowed down resulted in calcium influx through the PM (right peaks in Fig. 3B, 3C) due to the depletion of ER stores. These results were considered typical calcium imaging results for SOCE and were similar to thapsigargin (TG; 2 μ M)-induced calcium fluxes (Fig. 3A). Therefore, H₂O₂-induced calcium changes potentially occur mainly through SOCE. Furthermore, the inhibition of SOCE resulted in a significant decrease in cytosolic calcium in response to H₂O₂ insult (Fig. 3B, 3C).

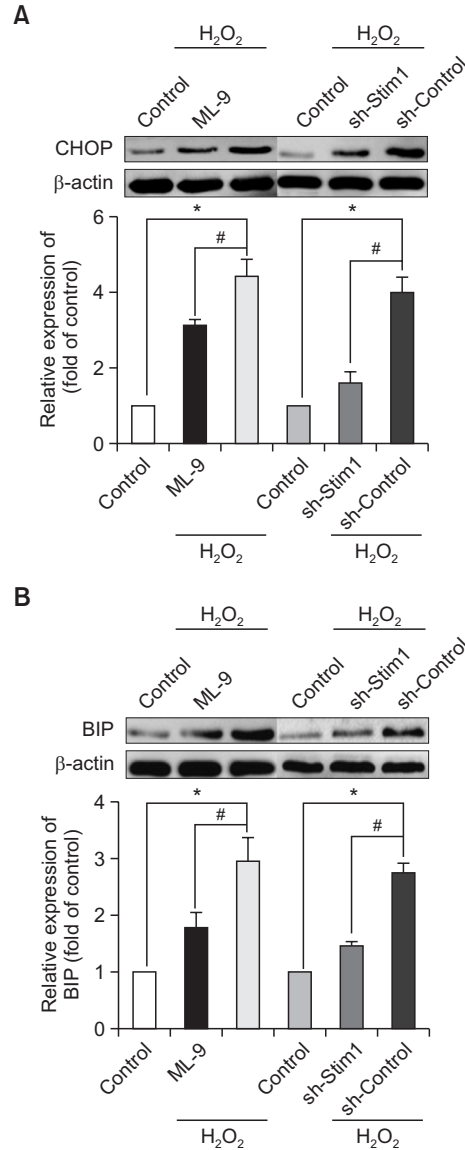


Fig. 5. Inhibition of SOCE protects EPCs from ER stress. EPCs that had been cultured for 7 days were pretreated with ML-9 for 20 min or with STIM1 shRNA before treatment with H₂O₂ for 6 h. The expression of the ER stress-related proteins CHOP (A) and BIP (B) was evaluated by Western blot analysis. * p <0.05; # p <0.05.

Inhibition of SOCE prevents intracellular ROS accumulation

To investigate the role of ROS in the protective effects of SOCE inhibition, we used the fluorescent probe DCFH-DA to detect intracellular ROS production. Compared with the control group, the H₂O₂ treatment group exhibited significantly increased ROS accumulation, and the inhibition of SOCE markedly blunted this effect (Fig. 4).

Inhibition of SOCE protects EPCs from ER stress

Many studies have demonstrated that ER stress is often accompanied by increased ROS production. To assess the protective effect of SOCE inhibition, we measured the expression of ER stress-related proteins. As shown in Fig. 5A, 5B, the expression levels of C/EBP homologous protein (CHOP) and

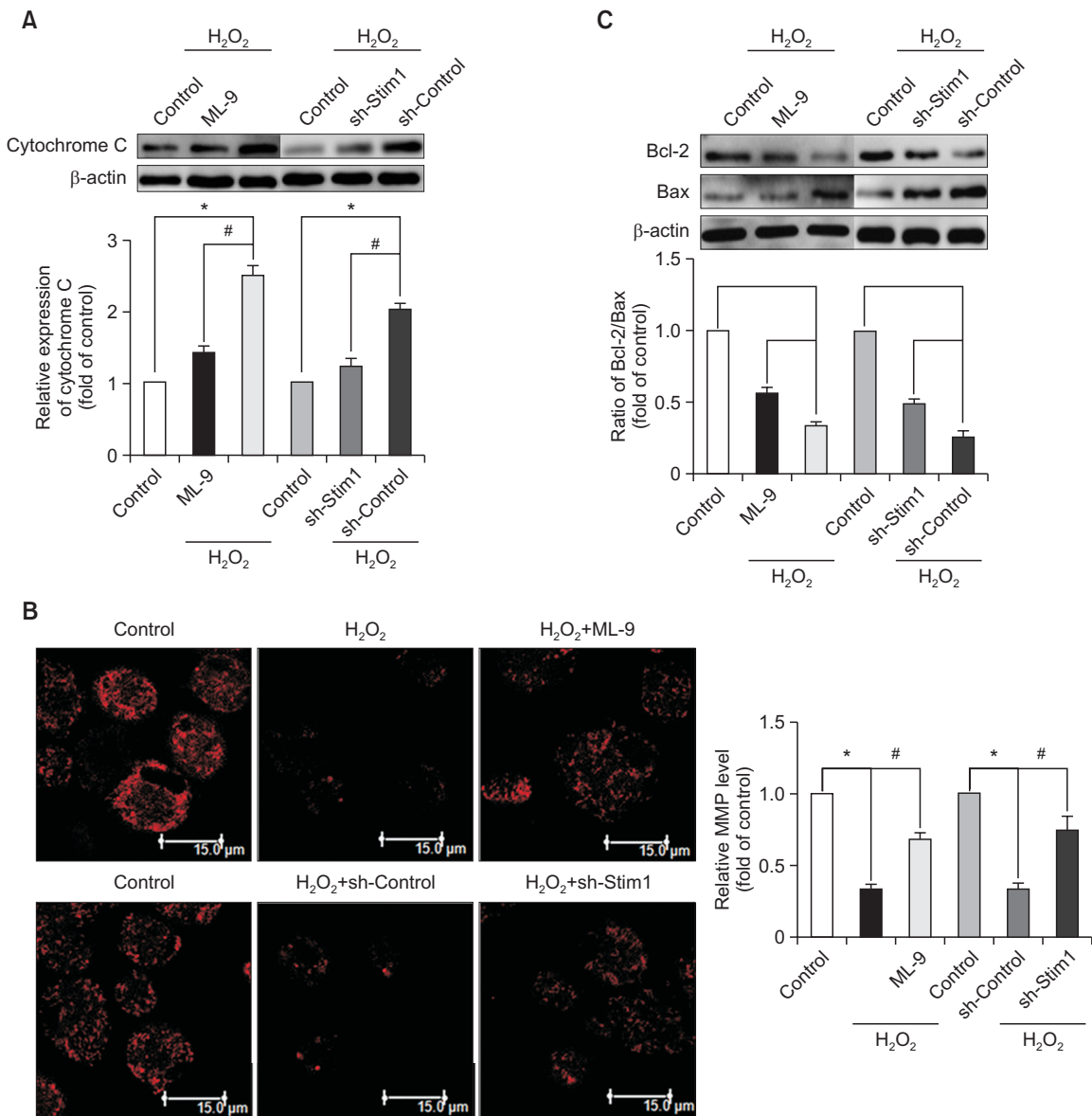


Fig. 6. Inhibition of SOCE ameliorates H₂O₂-induced mitochondrial dysfunction. EPCs that had been cultured for 7 days were pretreated with ML-9 for 20 min or with STIM1 shRNA before treatment with H₂O₂ for 6 h. The release of cytochrome C into the cytoplasm (A) and the Bcl-2/Bax ratio were measured by Western blot analysis (C). The MMP was measured via rhodamine 123 staining (B). **p*<0.05; #*p*<0.05.

CRP78/BIP were up-regulated by H₂O₂ stimulation compared with that of the control group. Moreover, the inhibition of SOCE suppressed the expression of ER stress-related proteins.

Inhibition of SOCE ameliorates H₂O₂-induced mitochondrial dysfunction

To demonstrate the effects of SOCE inhibition on the repair of mitochondrial dysfunction, we measured the MMP by Rh123 staining. After incubation with H₂O₂, the MMP was reduced compared with that of the control group, and this effect was ameliorated by SOCE inhibition (Fig. 6B). Moreover, the results of Western blot analysis suggested that H₂O₂ treatment up-regulated the cytosolic cytochrome C level compared with that of the control group (Fig. 6A). As predicted, SOCE inhibition significantly reduced the detrimental effects induced by

H₂O₂. Furthermore, we observed the effects of SOCE inhibition on Bcl-2 family members, which play a vital role in the mitochondrial pro-apoptotic signaling pathway. Exposure to H₂O₂ led to a decrease in the Bcl-2/Bax ratio compared with that of the control group (Fig. 6C), and this decrease was ameliorated by SOCE inhibition.

DISCUSSION

This study has shown for the first time that SOCE plays key roles in the H₂O₂-induced decrease in EPC cell viability and increase in EPC apoptosis. We have also demonstrated that SOCE inhibition attenuates H₂O₂-induced EPC cytotoxicity and apoptosis. First, H₂O₂ insult activated the process known

as calcium release-activated calcium influx. Second, SOCE inhibition suppressed H₂O₂-induced calcium influx. Third, SOCE inhibition attenuated H₂O₂-induced EPC cytotoxicity and apoptosis. Finally, SOCE inhibition significantly reversed the H₂O₂-mediated induction of several key pro-apoptotic signaling pathways mediated by ER stress and mitochondrial dysfunction. Taken together, our results demonstrate that SOCE inhibition protects EPCs from H₂O₂-induced apoptosis.

It is generally recognized that H₂O₂ insult leads to calcium influx and disruption of intracellular calcium homeostasis, followed by an increase in ROS production (Ohyama *et al.*, 2012). These changes further activate downstream apoptotic signaling pathways and ultimately result in cell death. Accumulating evidence suggests that oxidative stress-induced injuries might be attenuated by stabilizing intracellular calcium homeostasis (Godfraind, 2005; Ohyama *et al.*, 2012). Moreover, some studies have indicated that antioxidants diminish increases in intracellular calcium levels (Pieper and Dondlinger, 1998; Su *et al.*, 1999). Although the specific mechanism of the reduction of intracellular calcium following antioxidant application is still not clear, we hypothesized that the decrease in intracellular ROS production in response to antioxidant insult might lead to a change in the intracellular calcium level. In the present study, we demonstrated that H₂O₂ insult resulted in calcium influx and increase in intracellular ROS levels. In addition, we found that SOCE inhibition prevented intracellular ROS accumulation. To date, there have been no reports of the effect of antioxidants on calcium regulation in EPCs. Thus we will pay more attention to this aspect in our future study.

SOCE, which occurs ubiquitously in non-excitatory cells, plays an essential role in the regulation of intracellular calcium homeostasis. Accumulating evidence has demonstrated that SOCE is involved in oxidative stress (Törnquist *et al.*, 2000; Martín-Romero *et al.*, 2008; Henke *et al.*, 2013; Plomaritis *et al.*, 2014). However, the effect of SOCE on oxidative stress remains to be elucidated. Several studies have demonstrated that H₂O₂ insult weakens SOCE (Törnquist *et al.*, 2000; Plomaritis *et al.*, 2014). Other studies have shown that H₂O₂ insult augments SOCE and the influx of calcium ions (Rao *et al.*, 2013). These contradictory results may be explained by the activation of different signaling pathways in response to oxidative stress in different cell types. For example, the oxidative stress-induced changes in calcium influx and SOCE are mediated by the activation of protein kinase C (PKC) in thyroid FRTL-5 cells (Törnquist *et al.*, 2000) and by regulation of STIM1 expression in the HT22 cells (Rao *et al.*, 2013). In the present study, the H₂O₂ insult-induced disruption of intracellular calcium homeostasis was attenuated by inhibiting SOCE using ML-9 or via STIM1 silencing. These findings suggest that SOCE plays a critical role in oxidative stress.

Accumulating evidence supports the notion that calcium and calcium signaling are involved in ER and mitochondrial activities (Arnaudeau *et al.*, 2001; Pizzo and Pozzan, 2007). The ER, a site of major calcium stores, plays a critical role in a variety of cellular activities, such as protein synthesis, post-translational modifications, protein folding and apoptosis. A prolonged decrease in the ER calcium level (Hooper *et al.*, 2013) or accumulation of unfolded or misfolded proteins within the ER (Hammadi *et al.*, 2013) leads to ER stress. Prolonged ER stress further promotes the expression of CHOP, a CEBP family transcription factor, and caspase 12, thereby activating downstream apoptotic pathways. Moreover, ER stress can

result in sustained mitochondrial calcium accumulation (Deniaud *et al.*, 2008). The mitochondria, which are also important calcium-buffering organelles, are physically and functionally interconnected with the ER. Mitochondria are often located near calcium release sites in the ER or at calcium influx channels at the PM, where they buffer intracellular calcium changes by modulating the rate of capacitance calcium entry through calcium release-activated channels (CRACs) or the rate of calcium release by IP₃ receptors (Arnaudeau *et al.*, 2001). Disruption of mitochondrial calcium homeostasis may result in dysfunction of the mitochondria, resulting in up-regulation of caspase 9 expression and enhanced release of cytochrome c from the mitochondria into the cytoplasm. Cytochrome c and caspase 9 form apoptotic bodies through the multiple polymerization of apoptotic protease activating factor 1 (Apaf-1), thereby activating downstream apoptotic pathways (Hikita *et al.*, 2015). Several published reports have suggested that various apoptotic pathways are involved in the cross-talk between the ER and mitochondria (Wang and El-Deiry, 2004; Pizzo and Pozzan, 2007). Cytochrome c, which plays a vital role in this cross-talk, translocates to the ER to bind inositol 1,4,5-triphosphate receptors, leading to sustained calcium release. This calcium release in turn triggers the release of cytochrome c. Importantly, cytochrome c is involved in these apoptotic pathways. The present study demonstrated that SOCE inhibition restored intracellular calcium homeostasis and suppressed the pro-apoptotic effect by reducing ER stress and preserving mitochondrial function. Taken together, these data show that communication between the ER and mitochondria is involved in the protective effect of SOCE inhibition against H₂O₂ insult.

In conclusion, these findings have confirmed the role of SOCE in oxidative stress. H₂O₂-induced EPC apoptosis was shown to be alleviated by SOCE inhibition. The probable underlying mechanisms may be related to intracellular ROS levels, ER stress and mitochondrial dysfunction. Therefore, SOCE inhibition may serve as a potential therapeutic strategy for EPC injury in oxidative stress.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (grant 81370211). We would like to thank Prof. J.H.Z. and Prof. Y.Y. for the critical comments on this manuscript.

CONFLICTS OF INTEREST

All authors declare that there are no conflicts of interest.

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