Inhibition of Store-Operated Calcium Entry Protects Endothelial Progenitor Cells from H$_2$O$_2$-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 H$_2$O$_2$-induced apoptosis in endothelial progenitor cells (EPCs), which are the predominant cells involved in endothelial repair. The results showed that H$_2$O$_2$-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 H$_2$O$_2$. Our findings provide evidence that SOCE inhibition exerts a protective effect on EPCs in response to oxidative stress induced by H$_2$O$_2$ 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-excitable 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 Pentyala, 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.
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 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) following the manufacturer’s protocol. After 7 days of culturing, EPCs were treated with processing factors and then trypsinized and treated 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.

ShRNA transfection
STIM1 expression was silenced using a lentiviral vector carrying stromal interaction molecule 1 (STIM1) shRNA constructed 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 TTCTCGAAGCTGTCACGT. 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. 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.
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. 2. Inhibition of SOCE attenuates the detrimental effects of H$_2$O$_2$ 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 H$_2$O$_2$ 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.
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).

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 H\textsubscript{2}O\textsubscript{2} in a calcium-free solution, followed by treatment with 2 mM CaCl\textsubscript{2}. 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 H\textsubscript{2}O\textsubscript{2} for 6 h, and intracellular ROS levels were evaluated using a fluorescence probe (DCFHDA). *p<0.05; #p<0.05.
**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 μM) 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 μM, 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 μM) 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 μM), 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 μM)-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).

**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...
CRP78/BIP were up-regulated by H$_2$O$_2$ 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 H$_2$O$_2$-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 H$_2$O$_2$, 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 H$_2$O$_2$ 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 H$_2$O$_2$. 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 H$_2$O$_2$ 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 H$_2$O$_2$-induced decrease in EPC cell viability and increase in EPC apoptosis. We have also demonstrated that SOCE inhibition attenuates H$_2$O$_2$-induced EPC cytotoxicity and apoptosis. First, H$_2$O$_2$ insult activated the process known
as calcium release-activated calcium influx. Second, SOCE inhibition suppressed H$_2$O$_2$-induced calcium influx. Third, SOCE inhibition attenuated H$_2$O$_2$-induced EPC cytotoxicity and apoptosis. Finally, SOCE inhibition significantly reversed the H$_2$O$_2$-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 H$_2$O$_2$-induced apoptosis.

It is generally recognized that H$_2$O$_2$ insult leads to calcium influx and disruption of intracellular calcium homeostasis, followed by an increase in ROS production (Ohyma 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; Ohyma 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 calcium levels (Pieper and Dondlinger, 1998; Su et al., 1999) 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; Plomaritas et al., 2014). However, the effect of SOCE on oxidative stress remains to be elucidated. Several studies have demonstrated that H$_2$O$_2$ insult weakens SOCE (Törnquist et al., 2000; Plomaritas et al., 2014). Other studies have shown that H$_2$O$_2$ 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 H$_2$O$_2$ 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 capacitative 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 H$_2$O$_2$ insult.

In conclusion, these findings have confirmed the role of SOCE in oxidative stress. H$_2$O$_2$-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.

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**CONFLICTS OF INTEREST**

All authors declare that there are no conflicts of interest.

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