CSE/H_2S system protects mesenchymal stem cells from hypoxia and serum deprivation-induced apoptosis via mitochondrial injury, endoplasmic reticulum stress and PI3K/Akt activation pathways

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Received May 22, 2014; Accepted March 18, 2015

DOI: 10.3892/mmr.2015.3651

Abstract. Mesenchymal stem cells (MSCs) have the potential to facilitate cardiac repair following acute myocardial infarction. However, MSC therapy is limited by apoptosis of the stem cells following transplantation. Hydrogen sulfide (H_2S) has recently been proposed as an endogenous mediator of cell apoptosis in various systems. The aim of the present study was to investigate the mechanism underlying the antiapoptotic effect of the endogenous cystathionine γ-lyase (CSE)/H_2S system in MSCs cultivated in conditions of hypoxia and serum deprivation (H/SD). Western blotting was performed in order to determine the expression of proteins associated with the mitochondrial injury pathway, endoplasmic reticulum stress and the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. It was demonstrated that H/SD is able to significantly induce apoptosis in MSCs. CSE overexpression, which enhances the endogenous H_2S level, protects MSCs from H/SD-induced apoptosis via attenuation of the mitochondrial injury pathway, inhibition of endoplasmic reticulum stress and activation of the PI3K/Akt signaling pathway. In conclusion, the present findings suggest that modulation of the CSE/H_2S system may a therapeutic approach with which to promote the viability of transplanted MSCs.

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Key words: mesenchymal stem cells, hypoxia/serum deprivation, cystathionine γ-lyase/hydrogen sulfide, apoptosis

Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that are able to differentiate into cardiomyocytes and vascular endothelial cells (1). Therefore stem-cell therapy offers the prospect of a novel and effective treatment with which to repair ischemic heart tissue following acute myocardial infarction (AMI) (2). Injection of allogeneic MSCs into regions of damaged myocardium, 3 days after AMI has been shown to stimulate cardiac regeneration and to markedly decrease myocardial infarct size (3). The results from clinical trials have revealed that MSC engraftment via intramyocardial injection or intracoronary infusion is able to induce a moderate, but significant improvement in myocardial infarct size and left ventricular function (4). However, graft cell death is an important factor, which should be addressed in order to enable the development of cell therapy for cardiac repair. Ischemia and a hypoxic microenvironment may make the largest contribution to poor graft survival rate (5).

Hydrogen sulfide (H_2S) is a colorless, water soluble, flammable gas, which has a characteristic smell of rotten eggs. As with other members of the gasotransmitter family (nitric oxide and carbon monoxide), H_2S has been shown to possess extensive biological functions and may therefore be viewed as an important signaling molecule, involved in multiple signaling mechanisms under normal physiological conditions (6). Accumulating evidence suggests that exogenously applied H_2S and endogenously altered H_2S production are cytoprotective and regulate cell apoptosis in various models of cellular injury, including hypoxia (7), ischemia and reperfusion injury (8), oxidative stress (9) and inflammation (10). A previous study by this group demonstrated that hypoxia and serum deprivation (H/SD) is able to reduce endogenous H_2S production by inhibiting the expression and activity of cystathionine γ-lyase (CSE), a key enzyme involved in H_2S synthesis in MSCs (11). Upregulation of the CSE/H_2S system prevents the H/SD-induced decrease in endogenous H_2S generation and protects MSCs from apoptosis (11). However, the mechanism underlying the ability of endogenous H_2S

to protect MSCs from apoptosis under H/SD cultivation remains to be elucidated.

In the present study, a model of MSC apoptosis induced by H/SD was developed, and the overexpression of CSE in MSCs was achieved using lentivirus delivery, in order to examine the mechanisms underlying the antiapoptotic effect mediated by endogenous H_2S.

Materials and methods

Materials. Low-glucose Dulbecco's modified Eagle's medium (L-DMEM) and fetal bovine serum (FBS) were obtained from Hyclone (Logan, UT, USA). Propidium iodide (PI), RNase and DL-propargylglycine (PPG) were obtained from Sigma-Aldrich (St. Louis, MO, USA). A cell mitochondria isolation kit and trypsin-EDTA Solution were obtained from Beyotime Institute of Biotechnology (Haimen, China). Polyclonal rabbit CSE (sc-135203) and monoclonal mouse cytochrome *c* (sc-13561) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal Bax (BS6420) and rabbit polyclonal Bcl-2 (BS6421) antibodies were obtained from Bioworld Technology, Inc. (St. Louis Park, MN, USA). Rabbit polyclonal Akt (#9272), and rabbit polyclonal phospho-Akt (Ser473; #9271), rabbit polyclonal binding immunoglobulin protein (BiP; #3183) and rabbit polyclonal C/EBP homologous protein (CHOP; #2895) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The enhanced chemiluminescence western blotting system was purchased from EMD Millipore (Billerica, MA, USA). Lipofectamine 2000® was purchased from Invitrogen Life Technologies (Paisley, UK). Polybrene was obtained from Chemicon (Temecula, CA, USA).

Cell culture and model of H/SD. MSCs were isolated from Sprague-Dawley rats (30 male rats; 4 weeks old; ~80g; Shanghai Laboratory Animals Center, Shanghai, China) as previously described (11). Briefly, bone marrow was harvested from the tibia and femur of male rats, plated in L-DMEM supplemented with 20% inactivated FBS and 100 units/ml penicillin/streptomycin, and incubated at 37˚C in a humidified tissue culture incubator containing 5% CO_2. The medium was replaced after 24 h to discard non-adherent hematopoietic cells. The adherent spindle-shaped MSCs were expanded and cultured for no more than two or three passages. Subsequently, cells were analyzed for the expression of surface markers [CD44 and CD90 (positive), CD34 and CD45 (negative)], using flow cytometry (BD FACSCalibur; BD Biosciences, Baltimore, MD, USA) as described previously (12). All procedures performed on animals were approved by the University of Anhui Animal Care Committee (Hefei, China) and were conducted in accordance with national guidelines. Cell apoptosis was induced by H/SD, Briefly, MSCs were washed with serum-free L-DMEM, placed in serum-free medium and then incubated in a sealed hypoxic GENbox jar for 12 h fitted with a catalyst (GENbox anaer, Biomérieux, Marcy l'Etoile, France) in order to sequester free oxygen. The O_2 concentration was <0.1% after 2.5 h of using the GENbox anaer.

Plasmid construction, lentivirus production and transduction. Polymerase chain reaction was used to amplify the CSE gene (GenBank accession number, AY032875) from rat liver tissues using the following primer sequences: Forward, 5'-GTATGGAGGCACCAACAGGT-3' and reverse, 5'-GTTGGGTTTGTGGGTGTTTC-3' (Sangon Biotech, Co., Ltd., Shanghai, China). The cycling conditions were as follows: Initial denaturation at 95˚C for 5 min, 5 cycles of 95˚C for 30 sec, 63˚C for 30 sec (-1˚C/cycle) and 72˚C for 20 sec, 15 cycles of 95˚C for 30 sec, 58˚C for 30 sec (-0.5˚C/cycle) and 72˚C for 20 sec, then 19 cycles of 95˚C for 30 sec, 51˚C for 30 sec (-1˚C/cycle) and 72˚C for 20 sec. The amplified CSE gene was subcloned into the pLVX-IRES-ZsGreen vector using *in vitro* recombination methods. The pseudo-lentivirus was produced via transient transfection of 293FT packaging cells. On the day prior to transfection, $1.6x10^6$ 293FT cells were plated in 6-cm dishes. Subsequently, cells were cotransfected with either 1.7 μg pLVX-IRES-ZsGreen vector or pLVX-IRES-ZsGreen-CSE with all cells receiving 1.13 μg pCMV Δ8.91 and 0.57 μg pMD.G, using Lipofectamine 2000. The culture supernatants were harvested at 72 h following transfection and filtered through a 0.45-μm low protein binding polysulfonic filter (Millipore, Bedford, MA, USA). For transduction, $2x10^6$ MSC cells were seeded into a 10-cm dish and incubated with lentiviruses and 8 μg/ml polybrene in the incubator for 48 h.

Flow cytometry assay. Treated MSCs were digested with trypsin (2.5 g/l) and centrifuged at 250 x g for 5 min. The supernatant was then removed. Cells were washed twice with phosphate-buffered saline (PBS) and fixed with 70% ethanol at -20˚C overnight. Cells were then centrifuged at 250 x g for 5 min, washed twice with PBS and adjusted to a concentration of $1x10^6$ cells/ml. A quantity of 0.5 ml RNase (1 mg/ml in PBS; Sigma-Aldrich) was added into the 0.5 ml cell sample and incubated at 37˚C for 30 min. Following addition of the PI, to a final concentration of 50 mg/l, cells were filtered and incubated in darkness at 4˚C for 30 min, prior to flow cytometric analysis (Beckman-Coulter, Miami, FL, USA). In the DNA histogram, the amplitude of the sub-G1 DNA peak represents the quantity of apoptotic cells.

Cell mitochondria isolation. The mitochondria were isolated using a cell mitochondria isolation kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Briefly, $5x10^7$ cells were harvested and washed with ice-cold PBS. Cells were incubated with 1.0 ml mitochondria extraction mixed buffer provided in the kit for 15 min and then homogenized using an ice-cold dounce tissue grinder (Hede Biotechnology, Beijing, China). The homogenates were centrifuged at 600 x g for 10 min and then the supernatants were further centrifuged at 11,000 x g for 10 min at 4˚C. The supernatants were collected and the precipitate consisted of the cell mitochondria. The cytosolic proteins were isolated from the supernatant following further centrifugation at 12,000 x g for 10 min, at 4˚C. The samples containing the cell mitochondria were then separated using the mitochondria lysis mixed buffer for analysis of mitochondrial proteins.

Western blotting. Cultured cells were harvested and lysed. Equal quantities of proteins were boiled and separated by SDS-PAGE, then electrophoretically transferred onto a nitrocellulose

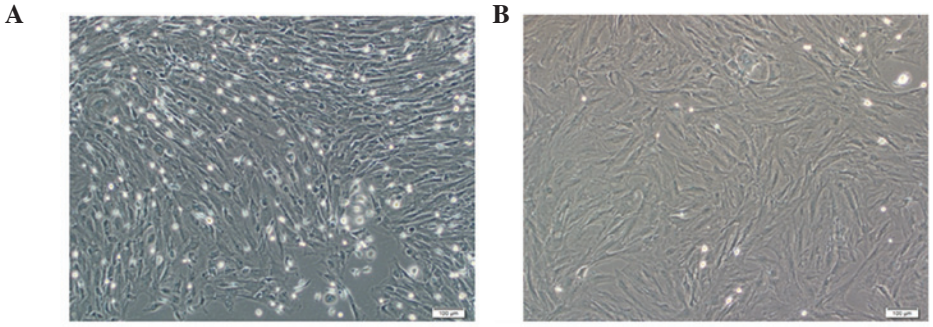

Figure 1. Phase contrast image of MSCs. (A) Representative image of MSCs at passage 0. (B) Representative image of MSCs at passage 3. (magnification, x10). MSCs, mesenchymal stem cells.

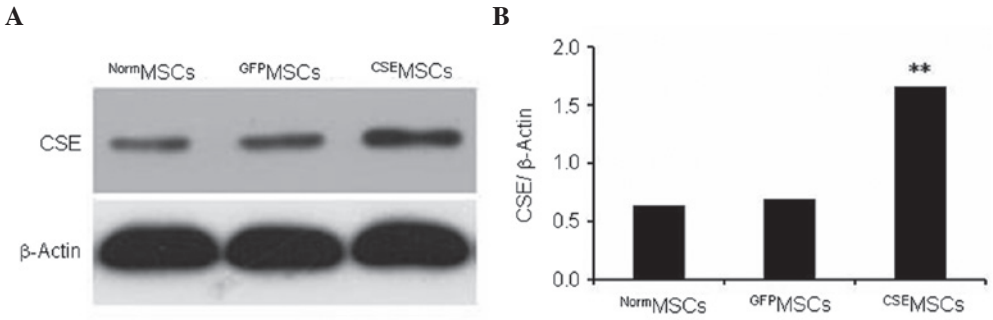

Figure 2. CSE overexpression mediated by lentiviral transduction. (A) Western blot analysis revealed higher CSE protein expression in ^{CSE}MSCs compared with ^{GFP}MSCs and ^{Norm}MSCs. (B) Blots were quantified by densitometry and plotted as the ratio of CSE to β-actin. Values are presented as the mean ± standard error of the mean from three independent experiments. **P<0.01 for ^{CSE}MSCs compared with ^{Norm}MSCs and ^{GFP}MSCs. MSCs, mesenchymal stem cells; CSE, cystathionine γ-lyase.

membrane (EMD Millipore). The membranes were blocked with Tris-buffered saline with Tween 20 (TBST) containing 5% bovine serum albumin (Sigma-Aldrich) for 2 h. The primary antibody dilutions were 1:500 for CSE, Bax, Bcl-2 and cytochrome *c*, and 1:1,000 for CHOP, BiP, Akt and p-Akt. The membranes were then incubated with primary antibodies at 4˚C overnight. Following washing with TBST, the membranes were incubated with goat anti-rabbit (#7074) or horse anti-mouse (#7076) horseradish peroxidase-conjugated IgG antibodies (Cell Signaling Technology, Inc., Beverly, MA, USA) diluted to 1:1,000 at room temperature for 2 h. The membranes were washed again and developed with an enhanced chemiluminescence system followed by apposition of the membranes with autoradiographic films (Eastman Kodak Company, Shanghai, China). The optical density of the protein band on western blots was calculated using Quantity One 1-D software, version 4.6.6 (Bio-Rad, Hercules, CA, USA).

Statistical analysis. Data are expressed as the mean ± standard error of the mean. Differences among groups were assessed using a one-way analysis of variance. Comparisons between the two groups were evaluated using post hoc tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Characteristics of cultured MSCs. MSCs were isolated and cultured from the bone marrow of male Sprague-Dawley rats. At 5 days following isolation, fusiform and fibroblast-like adherent cells were apparent, and formed cell colonies (Fig. 1). The determination of the surface markers of MSCs at passage 3 using flow cytometry, was the same as in a previous study by this group (12). The MSCs exhibited a positive expression of cluster of differentiation (CD)44, CD54 and CD90, while the expression of CD31, CD34 and CD45 was not observed.

Overexpression of CSE in genetically modified MSCs. CSE overexpression was mediated by lentiviral transduction in MSCs. The present data showed that CSE expression in MSCs infected with the pLV-ZsGreen-CSE lentivirus (^{CSE}MSCs) was upregulated by >2.5-fold compared with MSCs infected with the pLV-ZsGreen lentivirus (^{GFP}MSCs) or with untransduced MSCs (^{Norm}MSCs; Fig. 2).

Overexpression of CSE protects MSCs from H/SD-induced apoptosis in vitro. In order to further examine the regulatory role of CSE overexpression in H/SD-induced apoptosis in MSCs, the modified and normal MSCs were exposed to H/SD for 12 h. As shown in Fig. 3, it was observed that ^{CSE}MSCs had a significantly lower level of apoptosis compared with ^{Norm}MSCs or ^{GFP}MSCs. Therefore, the present data indicated that upregulation of the CSE/H_2S system protects MSCs from H/SD-induced apoptosis.

CSE protects MSCs from H/SD-induced apoptosis via inhibition of the mitochondrial injury pathway. Based on these initial results, the mechanism underlying the protection of MSCs from H/SD-induced apoptosis by CSE was further

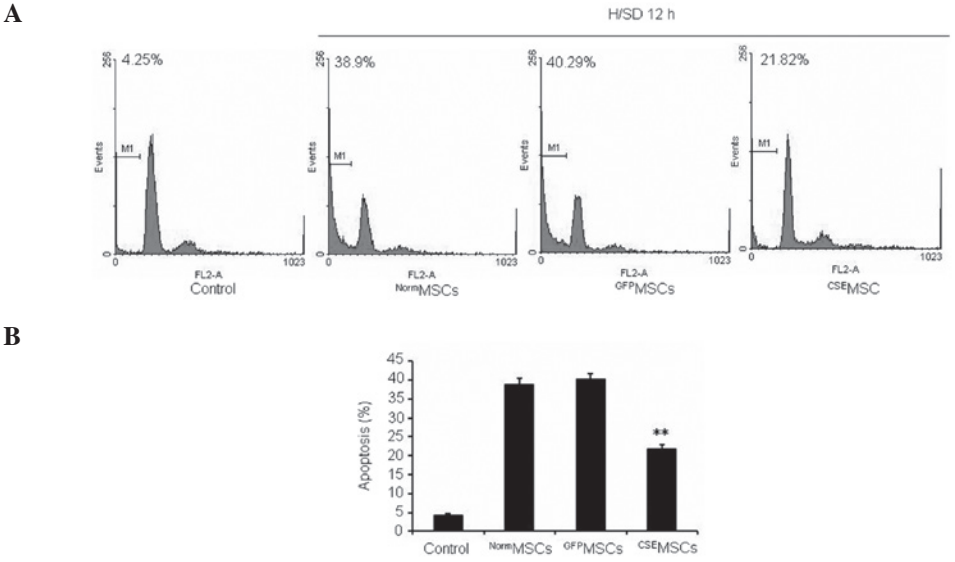

Figure 3. H/SD induces the apoptosis of MSCs. (A) MSCs were treated with H/SD for 12 h, stained with propidium iodide and analyzed using flow cytometry. (B) Quantitative analysis of the percentage of apoptotic cells. Values are presented as the mean ± standard error of the mean from three independent experiments. **P<0.01 for ^{CSE}MSCs compared with ^{Norm}MSCs and ^{GFP}MSCs. MSCs, mesenchymal stem cells; CSE, cystathionine γ-lyase; H/SD, hypoxia and serum deprivation.

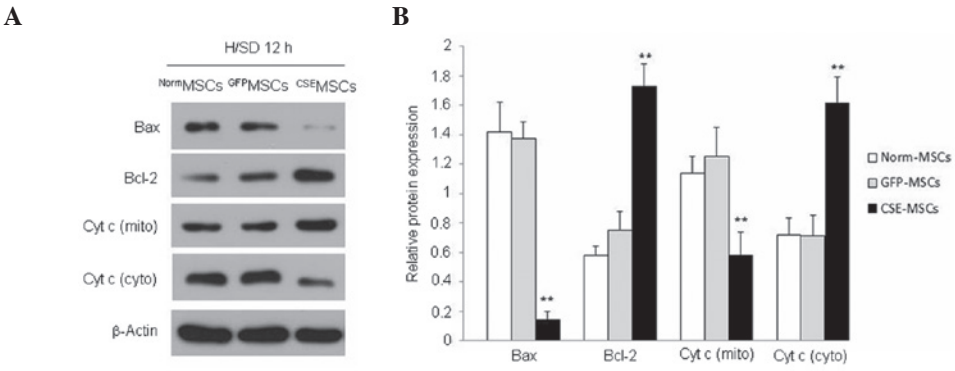

Figure 4. CSE overexpression attenuates mitochondrial injury in MSCs treated with H/SD. (A) Mitochondria injury-associated protein expression in MSCs was examined using western blotting. β-actin was used as an internal control. (B) Blots were quantified using densitometry and plotted as the ratio of protein of interest against that of β-actin. Values are presented as the mean ± standard error of the mean from three independent experiments. **P<0.01 for ^{CSE}MSCs compared with ^{Norm}MSCs and ^{GFP}MSCs. MSCs, mesenchymal stem cells; CSE, cystathionine γ-lyase; H/SD, hypoxia and serum deprivation; Cyt *c*, cytochrome *c*; mito, mitochondrial; cyto, cytosolic.

investigated. It has been reported that H/SD-induced apoptosis of MSCs is mediated by changes in the mitochondrial integrity and function, but may be independent of the death receptor pathway (13). Therefore, the role of the mitochondrial injury pathway was examined, primarily with regards to the protective effect of CSE overexpression against H/SD-induced apoptosis in MSCs. As shown in Fig. 4, following 12 h H/SD cultivation, the expression of Bax protein was reduced. However, Bcl-2 protein expression was increased in ^{CSE}MSCs compared with ^{Norm}MSCs and ^{GFP}MSCs. Changes in the level of cytochrome *c* in the cytosolic and mitochondrial fractions were also measured using western blotting. It was observed that cytochrome *c* was significantly increased in the cytosol but decreased in the mitochondria in ^{Norm}MSCs and ^{GFP}MSCs, compared with levels in ^{CSE}MSCs (Fig. 4). The present data demonstrated that H/SD cultivation promotes cytochrome *c* release from the mitochondria into the cytosol in MSCs. However, CSE overexpression may contain the cytochrome *c* within the mitochondria and inhibit the release of cytochrome *c* into the cytosol. The present data indicated that upregulation of the CSE/H_2S system inhibits mitochondrial injury and protects MSCs against H/SD-induced apoptosis.

CSE overexpression inhibits the H/SD-induced increase in CHOP and BiP expression in MSCs. Previous evidence has demonstrated that ERS is vital for cell apoptosis (14). The role of ERS has been investigated in multiple models of cell damage and apoptosis (15). It has been demonstrated that ERS is involved in H/SD-induced and H_2O_2-induced apoptosis of MSCs (16,17). In order to elucidate whether ERS was associated with the protective effect of CSE overexpression against H/SD-induced apoptosis in MSCs, the expression of two indicators of ERS (18), CHOP and BiP, was observed in genetically modified and normal MSCs. The result revealed that the expression of CHOP and BiP was downregulated in ^{CSE}MSCs subjected to H/SD for 12 h, compared with that in

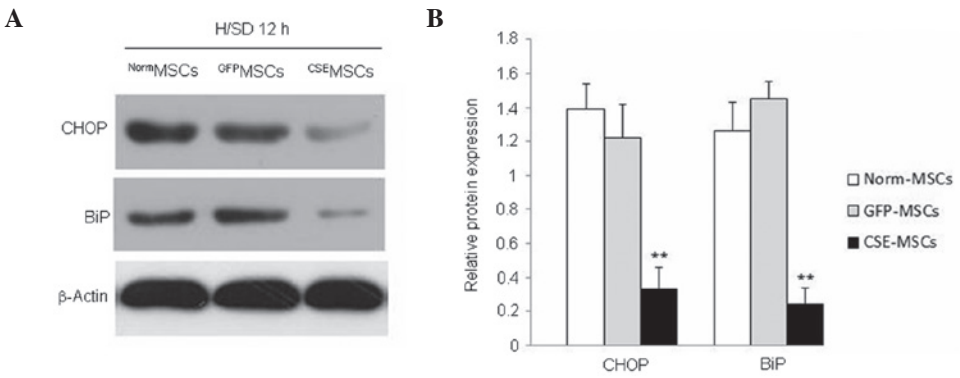

Figure 5. CSE overexpression inhibits endoplasmic reticulum stress in MSCs following H/SD treatment for 12 h. (A) Proteins involved in endoplasmic reticulum stress were examined using western blotting. β-actin was used as an internal control. (B) Blots were quantified by densitometry and plotted as the ratio of protein of interest against that of β-actin. Values are presented as the mean ± standard error of the mean from three independent experiments. **P<0.01 for CSEMSCs compared with NormMSCs and GFPMSCs. MSCs, mesenchymal stem cells; CSE, cystathionine γ-lyase; H/SD, hypoxia and serum deprivation; BiP, binding immunoglobulin protein; CHOP, C/EBP homologous protein.

NormMSCs or GFPMSCs (Fig. 5). These results indicated that the ERS response is inhibited by CSE overexpression and may be involved in the protective effect of endogenous H_2S on MSCs subjected to H/SD.

CSE overexpression activates the PI3K/Akt signaling pathway in MSCs. Overexpression of CSE promotes the phosphorylation of Akt in MSCs, and PI3K/Akt is an important cell survival pathway in various types of cells (19-21). Therefore, levels of total and phosphorylated Akt were measured using western blotting, in order to detect whether the overexpression of CSE activates the PI3K/Akt pathway. The results demonstrated that overexpression of CSE significantly increased the phosphorylation of Akt in CSEMSCs compared with that in NormMSCs or GFPMSCs (Fig. 6). In addition, H_2S production was inhibited following treatment of MSCs with the CSE inhibitor, PPG (5 mmol/l), in the presence of H/SD for 12 h. As shown in Fig. 6, PPG significantly inhibited the phosphorylation of Akt in CSEMSCs. These findings demonstrated that CSE overexpression activates the phosphorylation of Akt in MSCs subjected to H/SD. In addition, the CSE inhibitor, PPG, inhibits the phosphorylation of Akt in CSEMSCs following 12 h H/SD cultivation. Therefore, the present data indicated that endogenous H_2S protects MSCs from H/SD-induced apoptosis through the activation of the PI3K/Akt signaling pathway.

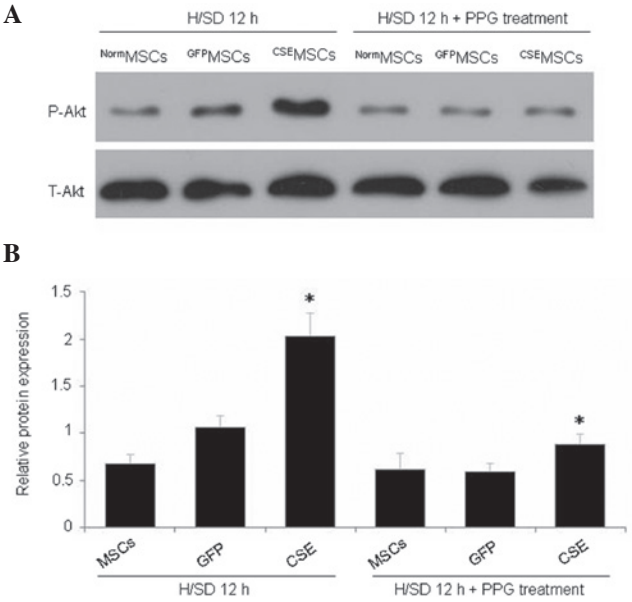

Figure 6. CSE overexpression activates the PI3K/Akt signaling pathway. (A) Level of phosphorylated Akt in MSCs when treated with PPG following exposure to H/SD for 12 h, were examined using western blotting. β-actin was used as an internal control. (B) Bands were quantified by densitometry and plotted as the ratio of protein of interest against that of β-actin. Values are expressed as the mean ± standard error of the mean from three independent experiments. *P<0.05 for CSEMSCs compared with NormMSCs and GFPMSCs. MSCs, mesenchymal stem cells; CSE, cystathionine γ-lyase; H/SD, hypoxia and serum deprivation; PI3K, phosphatidylinositide 3-kinase; PPG, DL-propargylglycine.

Discussion

The technique of *in vitro* cell culture has been widely utilized to imitate the ischemic microenvironment. The survival rate of MSCs in an ischemic microenvironment is critical to the success of cell-based transplantation therapy for ischemic heart disease. Therefore, it is important to elucidate the molecular mechanism responsible for the survival rate of transplanted MSCs.

It has previously been demonstrated that lysophosphatidic acid rescues ERS-associated apoptosis in H/SD-stimulated MSCs (16,22). Dong *et al* (23) observed that atorvastatin protects MSCs from H/SD injury via activation of an amp-activated protein kinase. Nie *et al* (24) found that the upregulation of microRNA (miR)-21, miR-23a and miR-210, induced by H/SD, may be involved in protecting MSCs from apoptosis (24). It has also been shown that heat shock protein 90 protects rat MSCs against H/SD-induced apoptosis via the PI3K/Akt and the extracellular-signal-regulated kinase (ERK)1/2 pathways (25).

After NO and CO, H_2S is considered to be the third identified endogenous 'gaseous transmitter' (26). Accumulating evidence has suggested that H_2S exerts protective effects against various ischemic and hypoxic injuries, in numerous tissues and cell models. It has been reported that H_2S protects HaCaT cells from cobalt chloride-induced cytotoxicity and inflammation, by negatively regulating the reactive oxygen species (ROS)/nuclear

factor-κB/COX-2 pathway (27). Yao *et al* (28) demonstrated that H_2S protects cardiomyocytes from hypoxia/reoxygenation-induced apoptosis by preventing glycogen synthase kinase (GSK)-3β-dependent opening of mitochondrial permeability transition pores (28). The K_{ATP}/PKC/ERK1/2 and PI3K/Akt pathways have been shown to contribute to H_2S preconditioning-induced cardioprotection (29). Elrod *et al* (30) observed that H_2S attenuates myocardial ischemia-reperfusion injury by preserving mitochondrial function. H_2S also protects endothelial cells from high glucose-induced apoptosis by inhibiting oxidative stress injury, leading to a decrease in intracellular ROS generation and malondialdehyde levels, and an increase in superoxide dismutase activity (31). Xie *et al* (32) revealed that exogenous H_2S preconditioning protects MSCs from hypoxia-induced cell death, an effect which was accompanied by a significantly increased level of phosphorylation of Akt, ERK1/2 and GSK-3β.

The signaling cascades that control caspase-dependent apoptosis may be classified into the mitochondrial injury and the death receptor pathways (33). The mitochondrial injury pathway may be induced by a wide variety of signals, including Bax and Bcl-2, amongst others, which result in the release of cytochrome *c* from the mitochondria into the cytoplasm. Bcl-2, as an antiapoptotic factor, is able to detain cytochrome *c* in the mitochondria, but Bax, as a proapoptotic factor is able to promote the release of cytochrome *c* into the cytoplasm. The endoplasmic reticulum (ER) is a multifunctional signaling organelle with sophisticated stress-signaling pathways that control the entry and release of Ca^{2+}, sterol biosynthesis, membrane protein translocation and apoptosis (34). GRP78, also known as BiP, is a critical ER chaperone protein, which performs multiple functions and is upregulated under conditions of stress to restore the function of the ER. CHOP exhibits low expression under physiological conditions, but is markedly induced in response to ERS. BiP and CHOP are accepted as markers of ERS (35). The PI3K/Akt pathway has been observed to respond to a variety of stimuli, including serum withdrawal, cell cycle disturbances, loss of cell adhesion and DNA damage, in a variety of cell types. In addition, the PI3K/Akt signaling pathway is important in mediating survival signaling in MSCs (36).

Using *in vitro* experiments, it was shown that 38.9% MSCs underwent apoptosis following H/SD for 12 h. By contrast, overexpression of CSE reduces the levels of apoptosis of MSCs by 21.82%. Compared with the control, the expression of Bax, CHOP and BiP was reduced. However, that of Bcl-2 increased. Additionally, cytochrome *c* remained in the mitochondria. Furthermore, it was found that overexpression of CSE promotes the phosphorylation of Akt, an effect which was eliminated following administration of the CSE inhibitor, PPG.

In conclusion, the present study demonstrated the harmful effect of H/SD on MSCs. The raised level of endogenous H_2S produced by CSE overexpression was shown to protect MSCs from H/SD-induced apoptosis, via negative regulation of the mitochondrial injury pathway, inhibition of ERS and activation of the PI3K/Akt signaling pathway. Therefore, the antiapoptotic effects of CSE and H_2S may be an effective approach to improve the cellular survival rate following cell-based therapy in transplantation.

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