Inhibition of the endogenous CSE/H$_2$S system contributes to hypoxia and serum deprivation-induced apoptosis in mesenchymal stem cells

CONGSHENG LI$^{1,2*}$, ZENG GUO$^{1*}$, BIRONG GUO$^{3}$, YANGJING XIE$^{1}$, JING YANG$^{2}$ and AILING WANG$^{1}$

$^{1}$Department of Cardiology, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui 230022; Departments of $^{2}$Emergency and $^{3}$Dermatology, The Third Affiliated Hospital of Anhui Medical University and the First People's Hospital of Hefei, Hefei, Anhui 230061, P.R. China

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Abstract. Mesenchymal stem cells (MSCs) have great potential for repair following acute myocardial infarction. However, a major challenge to MSC therapy is that transplanted cells undergo apoptosis. Hydrogen sulfide (H$_2$S) has recently been proposed as an endogenous mediator of cell apoptosis in various systems. The aim of the present study was to investigate the role of endogenous H$_2$S in hypoxia and serum deprivation (hypoxia/SD)-induced apoptosis in MSCs. The present study demonstrated that exposure of MSCs to hypoxia/SD caused a significant decrease in H$_2$S generation and resulted in marked cell apoptosis. Furthermore, under basal conditions, MSCs expressed cystathionine $\gamma$-lyase (CSE) and synthesized H$_2$S, whereas CSE expression and activity was inhibited by hypoxia/SD treatment. Overexpression of CSE not only markedly prevented hypoxia/SD-induced decreases in endogenous H$_2$S generation but also protected MSCs from apoptosis, while inhibition of CSE by its potent inhibitors significantly deteriorated the effect of hypoxia/SD in MSCs. These data indicate that the H$_2$S generation pathway exists in MSCs and the inhibition of the endogenous CSE/H$_2$S system contributes to hypoxia/SD-induced apoptosis in MSCs. Our findings suggest that modulation of the CSE/H$_2$S system is a potential therapeutic avenue for promoting the viability of transplanted MSCs.

Introduction

Acute myocardial infarction (AMI) is a major contributor to chronic heart disease leading to mortality in humans (1,2).

Transplantation of mesenchymal stem cells (MSCs) in the infarcted myocardium is considered to be a promising therapeutic option to repair the infarcted myocardium and restore the function of the damaged heart through multiple mechanisms (3-5). However, poor cell viability following transplantation significantly limits the therapeutic efficiency of MSCs (6,7). Mounting evidence has demonstrated that MSCs undergo apoptosis shortly following transplantation, which significantly reduces their effectiveness in tissue repair and regeneration (8,9). Thus, identifying the mechanisms associated with the apoptosis of MSCs and promoting the survival of transplanted MSCs may be vital for its successful utilization in cell therapy.

Hydrogen sulfide (H$_2$S), a poisonous gas used as a chemical reagent, has recently been proposed to be the third signaling gasotransmitter besides nitric oxide and carbon monoxide (10). In mammalian tissues, endogenous H$_2$S from L-cysteine is catalyzed primarily by two enzymes, cystathionine $\gamma$-lyase (CSE) and cystathionine $\beta$-synthase (CBS) (11). Accumulating evidence suggests that exogenously applied H$_2$S and inhibition of endogenous H$_2$S production regulate cell apoptosis in various systems, including the cardiovascular (12,13), nervous (14,15), respiratory (16,17) and pancreatic (18) systems. Xie et al (19) suggested that H$_2$S preconditioning protects MSCs against hypoxia and serum deprivation (hypoxia/SD)-induced apoptosis in vitro and enhances the efficacy of MSC transplantation in a rat model of AMI. However, the production of endogenous H$_2$S in MSCs and its regulatory effect on the apoptosis of MSCs are not yet completely understood.

In the present study, an apoptosis model induced by hypoxia/SD was established to determine whether H$_2$S could be endogenously generated by MSCs, and the role of endogenous H$_2$S in hypoxia/SD-induced apoptosis of MSCs was investigated.

Materials and methods

Materials. Low glucose Dulbecco’s Modified Eagle’s Medium (L-DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). L-cysteine, pyridoxal-5’-phosphate, propidium iodide (PI), RNase, DL-propargylglycine (PPG) and amino-oxyacetate (AOAA)
were provided by Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-rat β-actin polyclonal antibody and mouse monoclonal anti-CBS or -CSE antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The Enhanced Chemiluminescence (ECL) Western Blotting system was acquired from Millipore (Billerica, MA, USA). Lipofectamine 2000 was purchased from Invitrogen Life Technologies (Paisley, Scotland, UK) and polybrene was obtained from Chemicon (Temecula, CA, USA).

Cell culture and the model of hypoxia and SD. The MSCs were isolated from Sprague-Dawley rats (~4 weeks old, weight 80 g) obtained from the Laboratory Animal Center of Anhui Medical University (Hefei, Anhui, China) as previously described (20). All procedures in the present study were approved by the Animal Care and Use Committee of the First Affiliated Hospital of Anhui Medical University (Hefei, China). Briefly, bone marrow was harvested from the tibia and femur, plated in L-DMEM supplemented with 15% inactivated FBS and 100 units/ml of penicillin/streptomycin and incubated at 37°C in a humidified tissue culture incubator containing 5% CO₂ and 95% air. The medium was replaced 24 h later to discard nonadherent hematopoietic cells. The adherent, spindle-shaped MSCs were expanded and cultured for no more than two or three passages. Then they were analyzed by flow cytometry for the expression of surface markers as described previously (20). Apoptosis was induced by hypoxia/SD. Cells were washed with serum-free L-DMEM, placed in serum-free medium and then incubated in a sealed, hypoxic GENbox jar fitted with a catalyst (Biomay, Miami, FL, USA) to sequester free oxygen for 24 h. Oxygen tension in the medium, measured using a blood gas analyzer, was 33.5 mmHg within 0.5 h after being transferred into the hypoxic chamber and was maintained at approximately 22-24 mmHg throughout the experimental time.

Plasmid construction, transfection, production of lentivirus and transduction. PCR was used to amplify the CSE gene (GenBank™ accession number AY032875) from rat liver tissues using the following primer set: 5’-GTATGGAGGCCACAAACGTTT-3’ and 5’-GTGGTTGTTGGTTGGGTTTCT-3’. The amplified CSEgene was subcloned into the pLVX-ires-ZsGreen vector by in vitro recombination methods. A pseudo-lentivirus was produced by transient transfection of 293FT packaging cells (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China). One day prior to the transfection, 1.6x10⁶ 293FT cells were plated in 6-cm dishes. Then cells were cotransfected with either 1.7 µg of the pLVX-ires-ZsGreen vector or pLVX-ires-ZsGreen-CSE, 1.13 µg of pCMV Δ8.91 and 0.57 µg of pMD.G using Lipofectamine 2000. Culture supernatants were harvested 72 h after transfection and filtered through a 0.45 µm low protein binding polysulfonic filter (Millipore, Bedford, MA, USA). For transduction, 2x10⁵ MSC cells were seeded into 10 cm dishes and incubated with lentiviruses and 8 µg/ml of polybrene in the incubator for 48 h. Then the transduction efficiency was detected by the green fluorescence expression using an inverted microscope (TE2000-U; Nikon, Tokyo, Japan).

Flow cytometric analysis of apoptosis. Treated MSCs were digested with trypsin (2.5 g/l) and centrifuged at 250 x g for 5 min, and then the supernatant was 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 in PBS twice and adjusted to a concentration of 1x10⁶ cells/ml. A volume of 0.5 ml of RNase (1 mg/ml in PBS; Sigma-Aldrich) was added into 0.5-ml cell samples and incubated at 37°C for 30 min. Following mixing with PI (at a final concentration of 50 mg/l), mixed cells were filtered and incubated in the dark at 4°C for 30 min prior to flow cytometric analysis (BD FACS Calibur; Beckman Coulter, Miami, FL, USA). In the DNA histogram, the amplitude of the sub-G1 DNA peak represents the number of apoptotic cells.

Measurement of H₂S in the cell culture supernatant. The basis of the assay was that the H₂S produced in the incubate reacts with zinc acetate to form zinc sulfide, which then dissolves in a hydrochloric acid solution of N, N-dimethyl-p-phenylenediamine sulfate (NNDPD), yielding, in the presence of ferric chloride, methylene blue, which is quantitated spectrophotometrically. Cell culture supernatant (310 µl) was mixed with trichloroacetic acid (20% w/v, 60 µl), zinc acetate (2% w/v, 30 µl), NNDPD (20 mM, 40 µl) in 7.2 M HCl and FeCl₃ (30 mM, 30 µl) in 1.2 M of HCl. The absorbance of the resulting solution (670 nm) was measured 15 min thereafter by spectrophotometry (DU800; Beckman Coulter). H₂S concentration was calculated against a calibration curve of NaHS.

Assay of the activity of H₂S synthesizing enzymes. The MSCs were homogenized in 50 mM of ice-cold potassium phosphate buffer (pH 6.8). The reaction mixture contained 100 mM of potassium phosphate buffer (pH 7.4), L-cysteine (20 µl, 10 mM), pyridoxal 5'-phosphate (20 µl, 2 mM), saline (30 µl) and 11% w/v cell homogenate (430 µl). The reaction was performed in tightly stoppered cryovial test tubes and initiated by transferring the tubes from ice to an agitating water bath at 37°C. Following incubation for 30 min, 1% w/v zinc acetate (250 µl) was added to trap evolved H₂S followed by 10% v/v trichloroacetic acid (250 µl) to denature the protein and stop the reaction. Subsequently, NNDPD (20 mM; 133 µl) in 7.2 M HCl and FeCl₃ (30 mM; 30 µl) in 1.2 M HCl. The absorbance of the resulting solution (670 nm) was measured by spectrophotometry. The H₂S concentration was calculated against a calibration curve of NaHS, and H₂S synthesizing activity is expressed as nano-moles of H₂S formed per gram of protein (determined using the Bradford assay) per min (nmol/min/g protein).

Protein extraction and western blot analysis. For western blot analysis, cell lysates were prepared in the lysis buffer. Following centrifugation at 15,000 x g for 10 min, the supernatant was analyzed by western blotting. Total cell protein concentration was determined using the DC Protein Assay kit (Bio-Rad, Hercules, CA, USA). SDS-polyacrylamide gel electrophoresis was performed on 5% stacking and 12% resolving gel with low-range molecular weight standards (Solarbio, Beijing, China). Equal amounts of protein were loaded in each lane with loading buffer (Beyotime Biotechnology, Haimen, Jiangsu, China) containing 0.1 M Tris (pH 6.8), 20% glycerol, 10% mercaptoethanol, 4% SDS and 0.2% bromophenol blue. Following electrophoresis, the proteins were transferred to an
Immobilon-NC membrane (Millipore, Billerica, MA, USA). Following this, the membranes were blocked with Tris-buffered saline with Tween-20 (TBST; 50 mM of Tris-HCl, pH 7.4, 0.15 M of NaCl, 0.1% Tween-20) containing 5% BSA (Sigma-Aldrich) for 2 h. Following this, the membranes were incubated with primary antibodies at 4°C overnight. Following washing with TBST, the membranes were incubated with anti-rabbit IgG, HRP-linked antibody (CST; 1:1,000) at room temperature for 2 h. The membranes were washed again and developed with an ECL system (Kodak, Shanghai, China). The optical density of the protein band on western blots was calculated by Quantity One software (Bio-Rad).

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

Results

Hypoxia/SD induces cell apoptosis and inhibits endogenous H₂S production in MSCs. Flow cytometric analysis was used to investigate the effect of hypoxia/SD on apoptosis in MSCs. As shown in Fig. 1A and B, the treatment of MSCs with hypoxia/SD (3-24 h) significantly induced the apoptosis of MSCs in a time-dependent manner. At the same time, we evaluated whether hypoxia/SD affects the generation of endogenous H₂S in MSCs. Following exposure of MSCs to hypoxia/SD (3-24 h), the content of H₂S in culture supernatant was time-dependently decreased (Fig. 1C), indicating that hypoxia/SD was able to inhibit endogenous H₂S production in MSCs. These data imply that hypoxia/SD-induced apoptosis may be associated with the inhibition of endogenous H₂S generation in MSCs.

H₂S is produced by CSE in MSCs. CBS and CSE are the two key enzymes involved in H₂S formation from L-cysteine. Western blot analysis was used to investigate whether CBS and/or CSE are present in MSCs. As shown in Fig. 2A, CSE was detected in MSCs by western blot analysis; however, western blot analysis did not reveal the expression of CBS. This result indicated that CSE, but not CBS, is expressed in MSCs.

To confirm that CSE is the major enzyme responsible for endogenous H₂S generation in MSCs, we explored the effects of the inhibitor of CBS, AOAA, and the inhibitor of CSE, PPG, on H₂S synthesis in MSCs. H₂S synthesizing activity in MSCs was evaluated by the production of H₂S from added L-cysteine in the lysates of MSCs. The activity of H₂S synthesis in MSCs was significantly inhibited by pretreatment with PPG (Fig. 2B) for 30 min prior to the application of L-cysteine. By contrast, pretreatment with AOAA had no effect on the level of H₂S production (Fig. 2B), indicating that CSE, but not CBS, is involved in H₂S generation in MSCs.

Hypoxia/SD inhibits CSE expression and activity in MSCs. As shown in Fig. 3A and B, following treatment of MSCs with hypoxia/SD (3-24 h), CSE expression decreased in a time-dependent manner. Consistent with the results of CSE expression, the activity of CSE in MSCs was decreased by treatment with hypoxia/SD (3-24 h) in a time-dependent manner (Fig. 3C). These data indicate that inhibition of CSE expression and activity in MSCs contributes to the hypoxia/SD-elicted decrease in endogenous H₂S production.

CSE overexpression protects MSCs from hypoxia/SD-induced apoptosis in MSCs. To further explore the regulatory role of the endogenous CSE/H₂S system in hypoxia/SD-induced apoptosis in MSCs, we evaluated the effects of CSE overexpression on hypoxia/SD-induced decreases in endogenous H₂S generation and apoptosis in MSCs. CSE overexpression was mediated by lentiviral transduction in MSCs. Western blot analysis demonstrated that CSE expression in the MSCs infected by the pLV-ZsGreen-CSE lentivirus (CSEMSCs) was upregulated by more than 2.4-fold compared with the MSCs infected by the pLV-ZsGreen lentivirus (cuntMSCs) or untransduced MSCs (NormMSCs) (Fig. 4A and B). Next, the

Figure 1. Effects of hypoxia/SD on cell apoptosis and endogenous H₂S production in MSCs. (A) MSCs were treated with hypoxia/SD (3-24 h) and cell apoptosis was determined by flow cytometric analysis. (B) Quantitative analysis of the rate of apoptosis. (C) The content of H₂S in the culture supernatant was measured by the N,N-dimethyl-p-phenylenediamine sulfate method as described in Materials and methods. Values are the mean ± SEM of three independent experiments. *P<0.05, **P<0.01, compared with the control group. SD, serum deprivation; H₂S, hydrogen sulfide; MSCs, mesenchymal stem cells; H₂S, hypoxia/serum deprivation.
modified MSCs were treated under hypoxia/SD for 12 h. As shown in Fig. 4C, the content of H$_2$S in culture supernatant was significantly increased in the CSE/MSC group compared with the NormMSCs or GFPMSCs under hypoxia/SD for 12 h. Furthermore, we demonstrated that CSE/MSCs had a significant lower proportion of apoptosis (Fig. 4D and E) compared with NormMSCs or GFPMSCs groups under hypoxia/SD for 12 h. Overall, these data indicate that the upregulation of the CSE/H$_2$S system protects MSCs from hypoxia/SD-induced apoptosis in MSCs.

CSE inhibitor deteriorates hypoxia/SD-induced apoptosis in MSCs. To further confirm whether hypoxia/SD-induced apoptosis occurs via the endogenous CSE/H$_2$S system in MSCs, we inhibited H$_2$S production by applying the CSE inhibitor PPG in the presence of hypoxia/SD for 12 h. As shown in Fig. 5A, PPG (5 mmol/l) not only reduced H$_2$S generation but also exacerbated the inhibition of H$_2$S generation elicited by hypoxia/SD for 12 h. Furthermore, cell apoptosis (Fig. 5B and C) under hypoxia/SD was also significantly deteriorated by pretreating MSCs with the CSE inhibitor PPG (5 mmol/l) for 30 min. The CSE inhibitor PPG (5 mmol/l) treatment alone, however, had no effect on the apoptosis of MSCs (Fig. 5B and C). Overall, these data indicate that inhibition of the CSE/H$_2$S system deteriorates hypoxia/SD-induced apoptosis in MSCs.

Discussion

The present study demonstrated that CSE, but not CBS, was expressed in MSCs and involved in the endogenous generation of H$_2$S in MSCs. The treatment of MSCs with hypoxia/SD led to: i) time-dependent apoptosis in MSCs, ii) a decrease in endogenous H$_2$S generation, and iii) inhibition of CSE expression and activity. Furthermore, we also demonstrated that overexpression of CSE not only markedly prevented hypoxia/SD-induced decreases of endogenous H$_2$S production but also protected MSCs from apoptosis, while inhibition of CSE by its potent inhibitors significantly deteriorated the effect of hypoxia/SD in MSCs. Collectively, these findings suggest that hypoxia/SD induces apoptosis in MSCs by decreasing endogenous H$_2$S production via inhibiting the endogenous CSE/H$_2$S system.

Endogenous H$_2$S is now regarded as a novel signaling gasotransmitter and is important physiologically and pathologically in vivo and in vitro (21-24). MSCs, separated from bone marrow, peristeum, cord blood, skeletal muscle and adipose tissue, are capable of self-renewal (25) and multiple paths of differentiation (26) and have been demonstrated as an ideal cell source for the treatment of AMI (3 -5). Recent data demonstrated that H$_2$S was able to protect MSCs against hypoxia/SD-induced apoptosis in vitro and enhances the efficacy of MSC transplantation in a rat model of AMI (19). In the present study, we demonstrated that MSCs were able to generate H$_2$S and that this gaseous mediator is important in regulating hypoxia/SD-induced apoptosis. Two pyridoxal-5'-phosphate-dependent enzymes, CBS and CSE, are responsible for the majority of the endogenous production of H$_2$S in mammalian tissues (11). CBS is mainly expressed in the nervous system, whereas CSE appears to be predominant in the cardiovascular system (27). We demonstrated that CSE, but not CBS, was detected in MSCs and that conversion of L-cysteine to H$_2$S in the lysates of MSCs was inhibited by PPG, the inhibitor of CSE. These data suggest that CSE is the main enzyme involved in the generation of H$_2$S in MSCs. Recently, Shibuya et al (28) demonstrated that endogenous H$_2$S was also able to be produced by 3-mercaptopypyruvate sulftransferase (3-MST) along with cysteine aminotransferase in the brain. Further study needs to be carried out in...
the future to address whether MSCs are able to generate H$_2$S by catalyzing 3-MST.

Despite their several advantages for the treatment of AMI, a major challenge of MSC therapy is that transplanted cells undergo apoptosis (8,9) as they are exposed to an extremely harsh microenvironment in the infarcted heart. The efficiency of MSC transplantation is limited by the low viability of MSCs (29). Therefore, elucidating the molecular mechanisms underlying the apoptosis of MSCs may lead to important insights into the pathogenesis and treatment of AMI. H$_2$S deficiency was observed in atherosclerosis (30), ischemia-reperfusion injury (31), hypertension (32), gastric mucosal injury and liver cirrhosis (33). However, to the best of our knowledge, there is no information concerning H$_2$S generation in MSCs under hypoxia/SD conditions. In the present study, we demonstrated that the exposure of MSCs to hypoxia/SD led to a significant decrease in H$_2$S generation. We further demonstrated that hypoxia/SD inhibited the expression and activity of CSE in MSCs. These data indicate that hypoxia/SD reduces H$_2$S generation by inhibiting the expression and activity of CSE.

In the present study, we demonstrated that the treatment of MSCs with hypoxia/SD induced marked cell apoptosis in a time-dependent manner, which was consistent with the previous study (34). Taking into account the fact that hypoxia/SD decreases...
endogenous H$_2$S generation and inhibits CSE expression and activity in MSCs, we hypothesized that hypoxia/SD-induced MSCs apoptosis was associated with decreased endogenous H$_2$S production. To elucidate the contribution of decreases in endogenous H$_2$S to hypoxia/SD-induced MSC apoptosis, we investigated the effect of CSE overexpression and CSE inhibition on hypoxia/SD-induced apoptosis in MSCs. Our results demonstrated that overexpression of CSE not only markedly prevented hypoxia/SD-induced decreases in endogenous H$_2$S production but also protected MSCs from apoptosis in MSCs. Together with the aforementioned results that H$_2$S preconditioning protects MSCs against hypoxia and SD-induced apoptosis in vitro, we suggest that downregulating the endogenous CSE/H$_2$S system contributes to hypoxia/SD-induced MSC apoptosis. Furthermore, pretreatment with PPG, the inhibitor of CSE, caused a significant reduction in H$_2$S generation and deteriorated hypoxia/SD-induced apoptosis in MSCs, further confirming the contribution of decreases in endogenous H$_2$S production to hypoxia/SD-induced apoptosis in MSCs.

In summary, we have demonstrated that hypoxia/SD inhibits the expression and activity of CSE, a key enzyme for H$_2$S synthesis in MSCs. Overexpression of CSE is able to markedly prevent hypoxia/SD-induced decreases in endogenous H$_2$S generation and protect MSCs from apoptosis. Whereas, inhibition of CSE deteriorates the effect of hypoxia/SD in MSCs. These findings suggest that hypoxia/SD-induced apoptosis in MSCs occurs via the inhibition of the endogenous CSE/H$_2$S system. These data also establish a role for endogenous H$_2$S in the regulation of hypoxia/SD-induced apoptosis and identify the CSE/H$_2$S system as a novel therapeutic target for hypoxia/SD-induced apoptosis in MSCs.

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References