Hydrogen sulfide prevents H_2O_2-induced senescence in human umbilical vein endothelial cells through SIRT1 activation

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Abstract. The aim of the present study was to investigate the attenuation of endothelial cell senescence by H_2S and to explore the mechanisms underlying the anti-aging effects of H_2S. Senescence was induced in human umbilical vein endothelial cells (HUVECs) by incubation in 25 μmol/l H_2O_2 for 1 h. Senescence-associated β-galactosidase (SA-β-gal) activity was examined to determine the effects of H_2S on senescent HUVECs. The results indicated that SA-β-gal activity in the H_2O_2-treated HUVECs was 11.2±1.06%, which was attenuated in the NaHS group. Pretreatment with nicotinamide (NAM), a sirtuin 1 (SIRT1) inhibitor, inhibited the reduction in senescence associated with H_2S. Immunoblot analyses revealed that SIRT1 levels in senescent HUVECs treated with NaHS (60 μM) were indistinguishable from controls; however, analyses of SIRT1 activity indicated that SIRT1 enzyme activity was enhanced. In addition, we found that H_2S improves the function of senescent HUVECs. The present study demonstrated that H_2S protects against HUVEC senescence, potentially through modulation of SIRT1 activity. Furthermore, this study establishes a novel endothelial protective effect of H_2S.

Introduction

During aging, a number of physiological functions are altered and the cessation of cell division is accompanied by specific changes in cell function, morphology and gene expression. These changes may contribute to age-associated diseases, including hypertension, chronic coronary disease and diabetes (1). Vascular endothelial cells (VEC) are highly specialized and active cells exhibiting antithrombotic and anti-inflammatory properties. These cells are critically involved in the maintenance of vascular homeostasis by regulating vascular tone and integrity, as well as remodeling processes (2). Vascular cell senescence, which accompanies aging, promotes endothelial cell dysfunction (3) and is associated with increased vascular risk (4). Pathological states, including those observed in oxidative stress conditions, invoke irreversible growth arrest *in vitro* within a few days, a term referred to as stress-induced premature senescence (SIPS) (5,6). Previous evidence suggests that premature senescence of endothelial cells may lead to endothelial dysfunction and atherogenesis (3).

Hydrogen sulfide (H_2S) has attracted considerable interest as an endogenous gaseous mediator and potential pharmacological/therapeutic tool. It is endogenously generated from cysteine, in reactions catalyzed by cystathionine β-synthase (CBS) or cystathionine γ-lyase (CSE) (7). Studies in various species, including humans have demonstrated that H_2S is involved in diverse physiological and pathophysiological processes, including regulation of blood pressure (8), inflammation (9) and metabolic disorders (10). The beneficial effects of H_2S may be mediated through its antioxidant effects. H_2S is a potent inhibitor of O_2^- formation and gp91phox expression induced by TNF-α in pulmonary artery endothelial cells (PAECs) (11). Furthermore, H_2S protects endothelial cells against oxidized low-density lipoprotein (LDL) and hydrogen peroxide (H_2O_2)-mediated cell cytotoxicity (12). H_2S also appears to be a potent scavenger of oxygen-derived free radicals (13), which may contribute to the protective role of NaHS against the toxicity of H_2O_2 *in vitro* and *in vivo* (14). Findings of a previous study from our laboratory indicated that the endogenous CSE/H_2S system is downregulated in adipose tissues during aging (15). Another study reported that thermotolerance and lifespan of *Caenorhabditis elegans* (*C. elegans*) was increased when exposed to H_2S, which was mediated by SIR-2.1 activity (16).

SIR-2.1 is a *C. elegans* ortholog to sirtuin 1 (SIRT1), which is commonly known as nicotinamide adenine dinucleotide (NAD^+)-dependent class III histone deacetylase. This enzyme has been shown to modulate lifespan in yeast, worms, flies and mice. A mammalian SIRT1 homolog, silent information

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Abbreviations: H_2S, hydrogen sulfide; SIPS, stress-induced premature senescence; CSE, cystathionine γ-lyase

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regulator-2 (Sir2), is highly conserved in organisms ranging from archaea to humans and it has been shown to regulate cell cycle, senescence, apoptosis and metabolism by interacting with a number of molecules, including p53 (17) and Foxo1 (18). A previous study demonstrated that SIRT1 inhibition induces premature senescence-like growth arrest in human cancer cells (19). The aim of the present study was to investigate the effects of H_2S, using the donor NaHS, on the inhibition of H_2O_2-induced senescence in human umbilical vein endothelial cells (HUVECs) and the role of SIRT1 in this process.

Materials and methods

Materials. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), collagenase II, sodium hydrosulfide (NaHS) and DL-propargylglycine (PPG) were purchased from Sigma Aldrich (Zwijndrecht, Netherlands). Endothelial cell growth supplement (ECGS) was purchased from ScienCell Research Laboratories, Inc. (Carlsbad, CA, USA). p21 and SIRT1 antibodies and the Senescence β-Galactosidase Staining Kit were purchased from the Beyotime Institute of Biotechnology (Shanghai, China). Luminol reagent and polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (Billerica, MA, USA).

Cell cultures. HUVECs were isolated from newborn umbilical cords and cultured in M199 (Gibco, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS) and 2% EGCS at 37˚C under 5% CO_2 in a humidified atmosphere. Cells were used during passages two or three.

Immunohistochemistry. Immunohistochemical staining of the HUVECs for factor VIII-like antigen (fVIII-AGN) was performed on confluent cultures grown in 35-mm dishes. The media was aspirated and the cells were washed with phosphate-buffered saline (PBS) prior to fixing in bovine serum albumin (BSA) for 15 min at room temperature. The cells were washed again with PBS and incubated with a 1:40 (v/v) dilution of horseradish peroxidase (HRP)-conjugated rabbit anti-human fVIII:AGN (Bioss Inc., Woburn, MA, USA) for 45 min. The dishes were washed in triplicate with PBS, rinsed briefly with distilled water and mounted with buffered glycerol on glass cover slips. HRP-bound primary antibody was detected and observed using 3,3'-diaminobenzidine (DAB). Smooth muscle cells served as negative controls.

Senescence-associated β-galactosidase (SA-β-gal) staining. SA-β-gal activity was measured using a senescence cell staining kit. HUVECs were pretreated with various concentrations of NaHS, 5 mM NAM or a combination of NaHS and NAM for 48 h. The cells were then placed in media supplemented with 25 μM H_2O_2 for 1 h. The media were then replaced with normal medium and incubated for an additional 72 h. The cells were washed twice with PBS and the HUVECs were fixed and stained for SA-β-gal activity using the Senescence β-Galactosidase Staining Kit. The cells were then incubated at 37˚C for 16 h and SA-β-gal-positive cells were observed using microscopy, which included counting >400 cells in three independent fields. The percentage of SA-β-gal-positive cells was determined by counting the number of green cells within a sample (20).

Cell cycle assay. To determine the effect of H_2O_2 on cell cycle progression, HUVECs were grown for 1 h with or without 25 μmol/l H_2O_2. Cells were collected using trypsinization and centrifugation for 5 min at 300 x g and were fixed with 70% ethanol at 4˚C overnight. Cells were centrifuged to remove alcohol, stained with 50 mmol/l propidium iodide and washed twice with cold PBS. HUVECs were subjected to flow cytometric analyses with FACSCalibur and CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA). Cell cycles were analyzed and the proportion of cells in the G_0/G_1, S and G_2/M phases was recorded.

Immunoblot analyses. Protein extracts were prepared using the mammalian cell extraction kit following the manufacturer's instructions. Protein concentrations were determined using a BCA Protein Assay kit (Pierce, Biotechnology, Inc., Rockford, IL, USA). Extracted proteins were treated with 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, then heated at 100˚C for 10 min and separated using electrophoresis on a 10% SDS-polyacrylamide gel. Equal amounts of protein were separated using SDS-PAGE and then transferred to PVDF membranes. The membranes were incubated in a blocking buffer containing BSA (1%) and Tween-20 (0.1%, v/v) in Tris-buffered saline with Tween-20 (TBST) at room temperature for 2 h, and inoculated overnight at 4˚C with the primary antibodies, anti-human β-actin (1:500) and anti-human SIRT1 (1:300). The membranes were then inoculated with goat anti-rabbit (1:3,000) and goat anti-mouse (1:1,500) HRP-conjugated secondary antibodies at room temperature for 2 h. Each membrane was developed using enhanced chemiluminescence detection and quantified by densitometry.

Enzymatic activity assay. A SIRT1 enzyme activity assay was performed to determine the effect of H_2S on activity using a commercially available kit (Genmed, Plymouth, MN, USA). After preparing cell lysates, the SIRT1 activity assay was performed in a 96-well plate according to the manufacturer's instructions. The reaction product emitted fluorescence, which was detected using an excitation wavelength of 350 nm and an emission wavelength of 405 nm.

Cell proliferation assay. Proliferation of HUVECs was determined using an MTT assay (21). Forty-eight hours after cell seeding, the media were removed and 210 μl fresh culture media and 50 μl MTT solution (5 mg/ml in PBS) were added to each well, followed by incubation for 2 h at 37˚C in a 5% CO_2 atmosphere. The cells were cultured for 4 h at 37˚C in a 5% CO_2 atmosphere and the optical density of the solution was evaluated using a microplate spectrophotometer at 595 nm.

Cell scratch assay. To determine the functional consequences of senescence induced by H_2O_2, the *in vitro* scratch injury model was used. Cells were seeded in a 96-well plate and treated 24 h after seeding. Twenty-four hours after treatment, a thin-line, devoid of cells, was made by scratching the culture plate bottom with a 10 μl pipette tip. Following scratching, the

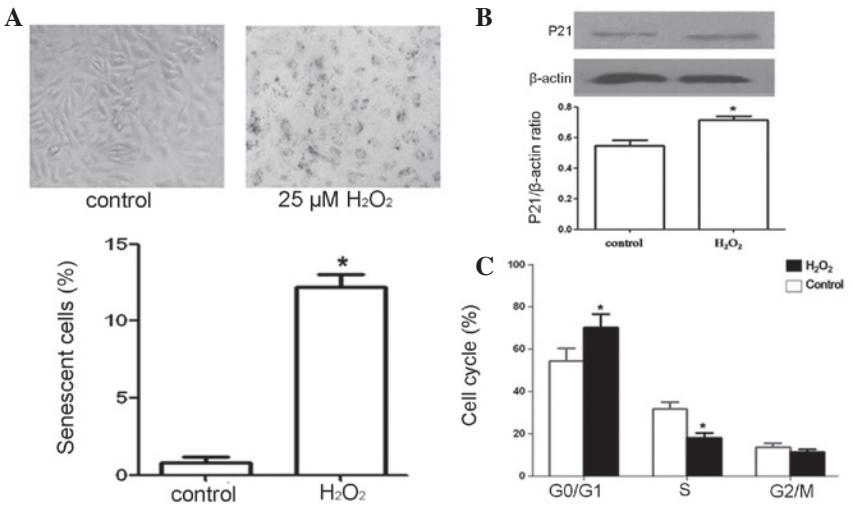

Figure 1. Low dose H_2O_2 induces senescence in human umbilical vein endothelial cells (HUVECs). (A) Senescence-associated β-galactosidase (SA-β-gal)-positive HUVECs increased significantly following treatment with 25 μM H_2O_2. (B) p21 protein levels in HUVECs following H_2O_2 treatment. Bars represent mean ± standard deviation (SD; *P<0.05 vs. control; n=3 experiments). (C) The effect of H_2O_2 on the cell cycle of HUVECs was arrested. *P<0.05 vs. control.

wells were washed with PBS and fresh media were added. Two images were captured per well; the width of the scratch was measured at four points per image with Image-Pro Plus and the means were calculated.

Statistical analyses. Results are expressed as mean ± standard error of the mean (SEM) or mean ± standard deviation (SD). Differences between groups were evaluated using analysis of variance, Dunnett's test or the least significant difference t-test. P<0.05 was considered to indicate a statistically significant difference.

Table I. Effect of H_2S on cell cycle arrest of HUVECs induced by 25 μM H_2O_2.

Group	G_0/G_1 (%)	S (%)	G_2/M (%)
Control	54.41±5.83	31.83±3.26	13.76±1.85
H_2O_2	70.24±6.31[a]	18.21±2.11	11.55±1.23
NaHS	58.16±5.34	29.08±2.52	12.91±1.36
H_2O_2+NaHS	53.72±5.12[b]	32.14±3.43	14.14±1.69

[a]P<0.05 vs. control group; [b]P<0.05 vs. H_2O_2 group (n=3). HUVECs, human umblical vein endothelial cells.

Results

Establishing a senescence model in HUVECs. To investigate the effect of H_2S on HUVEC senescence, we utilized an established senescence model, which involved incubating the cells with 25 μmol/l H_2O_2 for 1 h (22). Using light microscopy, we confirmed the presence of senescent HUVECs, which exhibited increased cell size and cytoplasmic granularity (Fig. 1A). The number of SA-β-gal-positive cells (Fig. 1A) and the proportion of HUVECs in the G_0/G_1 phase (Fig. 1C) were increased, indicating the presence of senescent cells. In order to confirm our results, we investigated p21 levels, which were increased in senescence (23). Immunoblot analyses indicated that p21 levels were increased in HUVECs treated with H_2O_2 (Fig. 1B). Collectively, these results indicate that the low concentration of H_2O_2 used in our study induced cell senescence.

H_2S protects against HUVEC senescence. SA-β-gal is a well-accepted biochemical marker of cell senescence (24). Examination of SA-β-gal activity in HUVECs treated with H_2O_2 (25 μM) revealed a significant increase in SA-β-gal-positive cells, which reached 11.2±1.06% (Fig. 1A). However, increases in SA-β-gal-positive cells were significantly attenuated in the NaHS (60 μM) group (Fig. 2A and B).

Progression through the cell cycle is a critical cellular process and cell cycle arrest during the G_1 phase is a characteristic exhibited by senescent cells. Our results demonstrated that treatment with 25 μM H_2O_2 arrested HUVECs in the G_0/G_1 phase as the proportion of cells in the G_0/G_1 phase was ~70.2% compared to 54.4% in the control group. NaHS (60 μM) pretreatment eliminated the effects of H_2O_2 and reduced the proportion of cells in the G_0/G_1 phase to 58.1% (Table I). These results indicate that H_2S protects against HUVEC senescence.

H_2S enhances the activation of SIRT1. To determine whether H_2S regulates HUVEC senescence through a SIRT1-mediated pathway, we examined the expression and activity of SIRT1. Immunoblot analyses indicated that SIRT1 levels were decreased in the H_2O_2 (25 μM) treatment group compared to the control, and NaHS (60 μM) treatment did not rescue SIRT1 expression (Fig. 3A and B). In contrast to its effect on protein expression, NaHS enhanced SIRT1 deacetylase activity *in vitro* (Fig. 3C), indicating a direct effect on SIRT1-mediated pathways. These results suggest that NaHS blocks senescence, cell differentiation and stress-induced apoptosis, and promotes cell growth by increasing SIRT1 deacetylase activity.

Inhibition of SIRT1 by NAM attenuates the anti-senescent effects of H_2S. To elucidate the role of SIRT1, HUVECs were pretreated with NaHS and/or NAM, a selective SIRT1

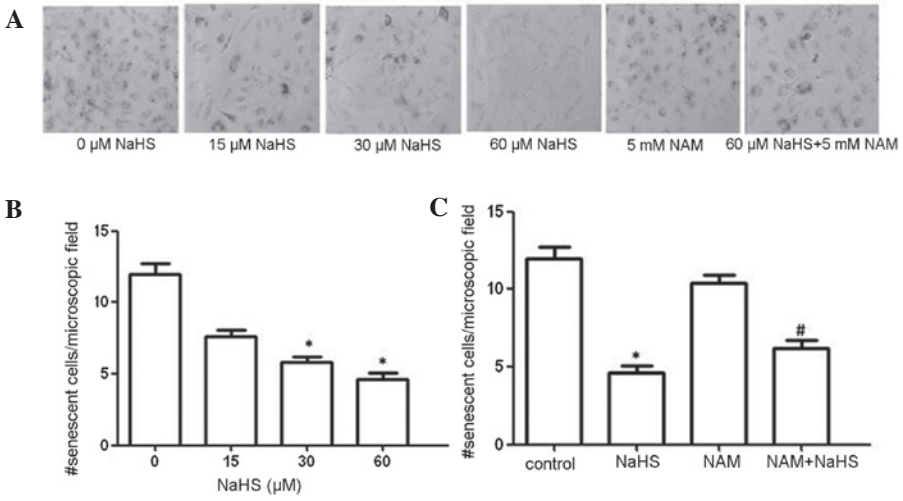

Figure 2. NaHS protects human umbilical vein endothelial cells (HUVECs) from senescence. (A) Images of senescence-associated β-galactosidase (SA-β-gal)-stained HUVECs are shown. Combinations of various doses of NaHS and/or nicotinamide (NAM) were administered 48 h prior to H_2O_2-induced senescence. (B and C) The number of SA-β-gal-positive cells per microscopic field 3 days following treatment. Values are presented as mean ± standard error of the mean (SEM) from three independent experiments. *P<0.05 vs. control; #P<0.05 vs. H_2O_2 group.

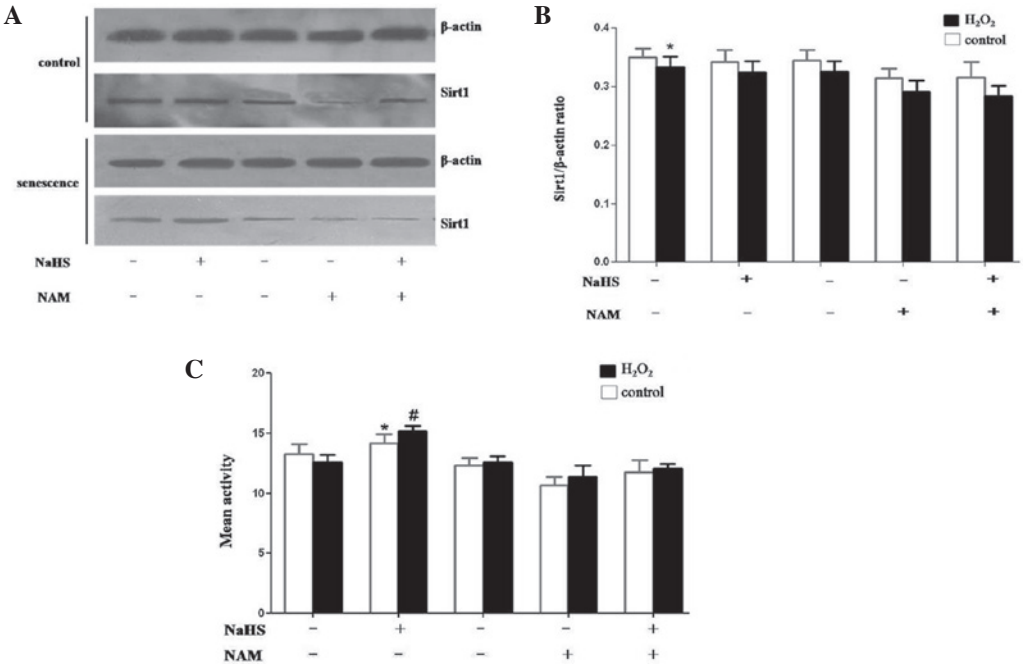

Figure 3. H_2S promotes sirtuin-1 (SIRT1) activity in human umbilical vein endothelial cells (HUVECs). SIRT1 expression was determined after combinations of NaHS (60 μM), nicotinamide (NAM; 5 mM) and NaHS were applied as a pretreatment 48 h prior to H_2O_2 administration in HUVECs. (A) SIRT1 expression in normal and senescent HUVECs. (B) SIRT1 protein expression was reduced in senescent HUVECs and treatment with NaHS did not attenuate this reduction. (C) SIRT1 deacetylase activity was reduced in the H_2O_2 treatment group and the addition of NaHS (60 μM) returned the activity to control levels. *P<0.05, #P<0.01 vs. control.

inhibitor, for 48 h prior to treatment with H_2O_2 (25 μM) for 1 h. NAM attenuated the decrease in SA-β-gal-positive cells inferred by NaHS alone (Fig. 2A and C).

H_2S prevents H_2O_2-mediated dysfunction in HUVECs. Since cell cycle arrest is a common hallmark of cellular senescence, we examined cell proliferation using the MTT assay. Our study indicated that NaHS (60 μM) improved H_2O_2-induced decreases in HUVEC proliferation. This reduction in proliferation was similar, but not significant, in cells pretreated with NAM and NaHS. Treatment with NaHS alone, however, was effective at rescuing the anti-proliferative effects of H_2O_2 (Fig. 4A). To further examine whether H_2S attenuates senescence-induced endothelial cell dysfunction, we monitored cell migration using a scratch assay (25). Cell migration was significantly reduced by H_2O_2 and pretreatment with NaHS eliminates this decrease. However, pretreatment with NAM and NaHS was associated with significantly decreased cell migration, which is similar to that observed in cells treated with H_2O_2 (Fig. 4C). We conclude that H_2S prevents the reduc-

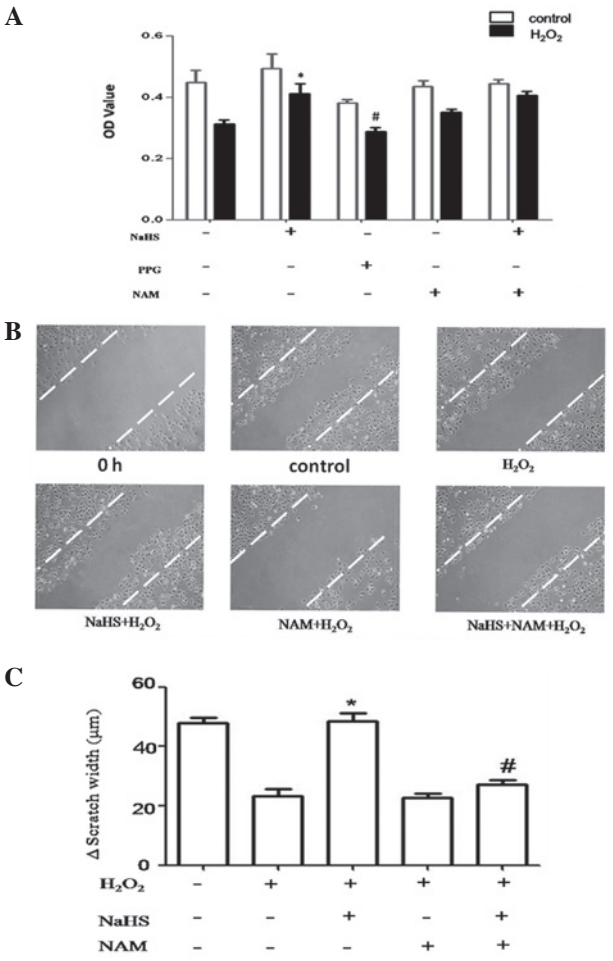

Figure 4. H_2S prevents H_2O_2-induced human umbilical vein endothelial cell (HUVEC) dysfunction. (A) Effects on cell proliferation were examined using the MTT assay and cell proliferation was calculated as the percentage of the controls (n=3); *P<0.05 vs. control. (B) Representative images showing the scratch at 24 h with various treatments. A mark was placed to locate the same area on the scratch. Images were obtained using an inverted microscope and the open area was calculated. (C) The migration width in different groups was calculated 24 h after treatment. Bars represent migration width in micrometers 24 h following scratching ± standard error of the mean (SEM; n=5 experiments); *P<0.05 vs. H_2O_2 group. The NaHS (60 μM) group had a higher migration rate compared to the controls.

tion in cell migration associated with senescence, an effect that is prevented by NAM. These results indicate that H_2S protects HUVECs against senescence through SIRT1.

Discussion

A number of studies have suggested that aging is an independent risk factor for the development of cardiovascular diseases. Previous studies further demonstrated that cellular senescence is involved in various pathological conditions, which are not limited to the cardiovascular system (26). Cellular senescence is a process by which cells irreversibly exit the cell cycle and cease to divide in response to a variety of stresses, including those observed during oxidative states (27). In this study, we established an H_2O_2-induced senescent model *in vitro* using HUVECs to investigate the protective role of H_2S in cell senescence.

Results of the present study demonstrated that a dose of 25 μM H_2O_2 increased the number of SA-β-gal-positive cells, which was eliminated following treatment with the H_2S donor, NaHS. These results demonstrated the importance of H_2S in preventing HUVEC senescence. The results obtained during cell cycle analyses were consistent with this observation. Coincidently, previous studies have shown that H_2S increases the lifespan in *C. elegans* (16). Our results suggest that H_2S may be responsible for retarding the aging process. Furthermore, we explored the mechanism of H_2S against HUVEC senescence.

The results of our study indicated that SIRT1 expression was unchanged after HUVECs were pretreated with 60 μM NaHS for 48 h, while SIRT1 enzyme activity was enhanced, indicating that SIRT1 is a key sensor system for regulating endothelial cell survival, proliferation and senescence. A recent study suggested that SIRT1 overexpression in a mouse model led to a significant improvement in animal health during aging (28). Another study demonstrated that the protective effects of SIRT1 may be due to the regulation of acetylation/deacetylation of key proteins (29). One study indicated that SIRT1 protein levels decrease rapidly with each increase in cell passage and this leads to premature senescence (30). SIRT1 protein stability may play a role in the progressive loss of SIRT1 associated with aging; however, the mechanisms remain elusive. One study indicated that post-translational modification by sumoylation affects the activity of SIRT1 (31). Another study demonstrated that ~10-25% of liver proteins, including actin, tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), undergo sulfhydration under physiological conditions. Sulfhydration appears to be a physiological post-translational modification for proteins (32). We consider that H_2S may activate SIRT1 via sulfhydration of the SIRT1 protein. It is possible that SIRT1 is indirectly activated via other H_2S-induced physiological alterations. The hypothesis that H_2S shifts redox homeostasis, thereby increasing available NAD^+ (or the NAD^+/NADH ratio) and resulting in increased SIRT1 activity is controversial.

HUVEC senescence and the consequent reduction of their proliferative and migration ability may contribute to miopragia associated with advanced age. In the present study, we observed that HUVECs treated with 25 μM H_2O_2 have a reduced ability to migrate, while NaHS treatment prevented this process. However, the mechanisms by which H_2S improves cell proliferation remain unclear. It is accepted that increased cellular senescence is associated with decreased cell proliferation *in vivo* since senescent cells are not able to divide (33). Therefore, it may be concluded that NaHS (60 μmol/l) improves HUVEC proliferation by delaying cell senescence.

Exogenous H_2S has notable effects on mammalian physiology that improve survival in changing environmental conditions (17). Despite the molecular mechanisms involved in the modulation of SIRT1 activity, H_2S affects SIRT1 activity and attenuates senescence, thus establishing that H_2S exhibits novel endothelial protective effects. FoxO1, is a downstream target of SIRT1, which has been shown to modulate G_1-S and G_2-M phase transition by coordinating the expression of multiple important cell cycle regulators (34). In future studies, we aim to evaluate the effect of H_2S on FoxO1 via SIRT1 expression and activity.

A previous study indicated that H_2S-releasing diclofenac derivatives, a novel class of non-steroidal anti-inflammatory drugs (NSAIDs), may be of clinical value in the treatment of osteolytic bone disease (35). In addition, preclinical studies have indicated that the novel hydrogen sulfide-modulating agent, S-propargyl-L-cysteine (SPRC) is a potent cardioprotective candidate (36). Our study provides a novel therapeutic role for H_2S, which protects against HUVEC senescence.

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