Exogenous H_2S Regulates CSE Expression in HUVECs under Hypoxic Conditions

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Abstract

Hydrogen sulfide (H_2S) is a gaseous signaling molecule synthesized in the body by three enzymes: cystathionine-beta-synthase (CBS), cystathionine-gamma-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST). CSE plays a crucial role in the production H_2S in the cardiovascular system. The primary objective of this study was to investigate the effects of exogenous H_2S on CSE expression in vascular endothelial cells under hypoxic conditions. Using luciferase assays, quantitative RT-qPCR, and western blotting, we conducted a comprehensive analysis of the regulatory effects of 100 μM H_2S on CSE transcription and expression in human umbilical vein endothelial cells (HUVECs) subjected to hypoxia. This study specifically examined the impact of exogenous H_2S on CSE promoter activity in HUVECs. Notably, compared to 0 μM H_2S, exposure to 100 μM H_2S resulted in a significant reduction in mRNA and CSE protein expression in HUVECs following a two-hour hypoxic period. The administration of exogenous H_2S further diminished CSE expression at various time points in HUVECs under hypoxic conditions. Technical terms were defined based on their initial applications. These findings strongly suggest that vascular endothelial cells respond to fluctuations in blood H_2S levels during oxygen-deficient periods.

Keywords: Hydrogen sulphide, Cystathionine-γ-lyase, Hypoxia, HUVECs

1. Introduction

Over the past few years, hydrogen sulfide (H_2S) has been recognized as an important endogenous regulator of physiological processes, despite its noxious smell, reminiscent of rotten eggs, toxic nature, and potential environmental risks [1]. Cystathionine γ-lyase (CSE), cystathionine-β-synthase (CBS), cysteine aminotransferase (CAT), and 3-mercaptopyruvate sulfur transferase (3-MST) are the major synthetases of hydrogen sulfide (H_2S) [2]. H_2S is produced in the vasculature and promotes vascular homeostasis, vasodilation, and endothelial cell proliferation [3]. After inhibiting endogenous CSE expression, direct administration of 100 μM exogenous H2S induced apoptosis in human aortic smooth muscle cells [4]. Mice overexpressing CSE in the heart exhibit resistance to ischemia-reperfusion injury, accompanied by decreased myocardial inflammation [5]. Dysregulation of CSE expression in skeletal muscles contributes to metabolic disorders associated with high-fat diet (HFD) [6]. Vascular smooth muscle cells (SMCs) from CSE

gene-knockout mice are more susceptible to apoptosis induced by exogenous H_2S at physiologically relevant concentrations than those from wild-type mice [7]. High levels of homocysteine induce endothelial cell dysfunction, and the metabolism and physiological functions of H_2S enable it to function as a protective agent [8]. H_2S is a signaling molecule and cytoprotectant that protects various tissues and organs from oxidative stress and ischemia-reperfusion injury [9]. Endogenous H_2S plays a modulatory role in hypoxia-induced cardiovascular responses and inhibits cardiovascular disease in spontaneously hypertensive rats (SH) [10]. Endothelial CSE contributes to cardiovascular homeostasis primarily through the production of H_2S [11]. Thus, GYY4137 (a slow-releasing H2S donor) may be a novel therapeutic tool for preventing diabetes-associated vascular dysfunction [12]. H2S ameliorates left ventricular diastolic dysfunction by restoring mitochondrial abnormalities via upregulating PGC-1alpha and its downstream targets NRF1 and TFAM. Modifying H_2S signaling is a potentially

novel therapeutic approach for the management of hypertension; however, further experimental clinical studies on the role of H_2S in hypertension are required [13]. The CSE/H_2S system is an important therapeutic target for protection against renal ischemia/reperfusion injury (IRI), and it may protect renal tubule epithelial cells from IRI by suppressing endoplasmic reticulum stress (ERS)-induced autophagy [14]. A decrease in the activity of H_2S-producing enzymes and H_2S levels may contribute to oxidative stress by decreasing the activity of H_2S-producing enzymes, and H_2S levels may contribute to oxidative stress in PCOS. [15]. Aging leads to a decrease in H_2S levels in the heart and plasma of mice and severe impairment of cardiac diastolic function, interstitial relaxation, and fibrosis of the heart [16]. CSE regulation of tumor necrosis factor-alpha (TNF-α)-controlled intracellular signaling pathways could provide new therapeutic targets for colon diseases associated with impaired epithelial wound healing [17]. The administration of NaHS curtailed cardiomyocyte pyroptosis and augmented cell viability, and additional studies have indicated that the mitigating effect of H_2S on cardiomyocyte pyroptosis is modulated through the ROS/NLRP3 pathway [18]. Endogenous H_2S influences the pathogenesis of acute kidney injury (SAKI), whereas exogenous H_2S protects against LPS-induced AKI by inhibiting the PERK/Bax-Bcl2 pathway involved in endoplasmic reticulum stress (ERS) [19]. The CSE/H_2S signaling pathway may be a potential therapeutic target for the treatment of liver diseases [20]. Ferroptosis presents a significant challenge in mesenchymal stem cell (MSC)-based therapies. Hence, the emerging role of CSE/H_2S signaling in abrogating ferroptosis provides a novel option for therapeutic intervention [21]. The CSE/H_2S system maintains lipid homeostasis and cellular senescence in heart cells under lipid overload [22]. Abolishing the H_2S-synthesizing machinery, particularly via miR-30a-5p, may represent a promising therapeutic strategy for patients with TNBC [23].

Several studies have investigated the effects of H_2S on human blood vessels. The H_2S signaling pathway and cystathionine gamma-lyase (CSE), which is responsible for H_2S generation, have been identified as key regulators of vascular function [24]. H_2S-induced relaxation has been demonstrated in the internal mammary, pulmonary, mesenteric, and intrarenal arteries, and perfused human placentas [25-29]. The upregulation of CSE expression under hypoxic conditions may increase the production and concentration of H_2S in cells and protect them from hypoxia [30]. A controlled-release formulation of S-propargyl-cysteine exerts protective effects against myocardial infarction (MI) via the CSE/H_2S pathway [31]. NADPH oxidase 4 is a positive transcriptional regulator of CSE in endothelial cells, and some researchers have proposed that it modulates endogenous H_2S production [32]. CSE-derived H_2S production by endothelial cells is critical for maintaining endothelial function and exercise capacity and protecting against myocardial ischemia/reperfusion injury [33]. H_2S has the potential to restore the aging-induced loss of cardioprotective effects of remote ischemic preconditioning (RIPC) by upregulating HIF-1alpha/Nrf2 signaling [34]. Sodium hydrosulfide (NaHS), an H_2S donor, restores vascular function in streptozotocin-induced hyperglycemia (HG) via renin-angiotensin

system (RAS) modulation [35]. In HUVECs, IRE1alpha-JNK induced autophagy is involved in hyperhomocysteinemia (HHcy)-induced endothelial dysfunction, whereas NaHS stimulation reversed the protein expression in the IRE1alpha/JNK-autophagy pathway with Hcy incubation [36]. S-propargyl-cysteine (SPRC, an endogenous H_2S donor) may serve as an aging-protective agent, and pharmacological targeting of Jumonji domain-containing protein 3 (JMJD3) may also be a promising therapeutic approach for age-related heart diseases [37]. Exogenous H_2S supplementation alleviated the proliferation of skin fibroblasts upon TGF-β (1) stimulation via necroptosis inhibition [38]. Increased production of H_2S by CSE is at least partially responsible for tumor vascular normalization, leading to decreased leakiness and enhanced delivery of chemotherapeutic agents to the tumor [39].

H_2S has been identified as an excitatory mediator of hypoxic sensing in the carotid body [40]. Incubation with NaHS increases the expression of miR-21 and attenuates the reduced cell viability and increased apoptosis caused by ischemia-reperfusion (I/R) in BRL cells [41]. An in vitro study showed that an exogenous H_2S donor attenuated hypoxia-induced apoptosis in primary rat nucleus pulposus (NP) cells [42]. Exogenous administration of NaHS may be a potential strategy for treating Ni-induced lung cancer progression [43]. Pretreatment with NaHS or aspirin (ATB-340) in aged rats fed a high-fructose diet (HFD) and exposed to water-immersion restraint stress (WIRS) attenuated gastric damage compared to vehicle treatment [44]. Both endothelial H_2S, mainly catalyzed by CSE, and exogenous H_2S protect HNCs against hypoxia-reoxygenation injury via RhoA Ser188 phosphorylation [45]. Endogenous H_2S protects against vascular remodeling by preserving the PPAR delta/SOCS3 anti-inflammatory signaling pathway [46]. H_2S can alleviate hypothyroidism-induced myocardial fibrosis by activating autophagy and suppressing the TGF-beta1/SMAD family member 2 (Smad 2) signal transduction pathway [47]. NaHS supplementation mitigates hyperhomocysteinemia (Hhcy)-induced liver injury by downregulating hepatic autophagy through S-sulfhydration and activating serum and glucocorticoid-regulated kinase 1 (SGK1). This post-translational modification by H_2S holds promise as a therapeutic approach for HHcy-induced liver injury [48]. Pharmacological H_2S supplementation improves diastolic function and reduces cardiac fibrosis in heart failure with preserved ejection fraction (HFpEF) models [49]. Endogenous CSE/H_2S in Vascular smooth muscle cells (VSMCs) reduces VSMC senescence and stiffness, thereby attenuating arterial stiffness and aging, partly through sulfhydration-mediated activation of Foxm1 and subsequent inhibition of Gas1 signaling pathways [50].

Some studies have indicated that optimal levels of exogenous H_2S can affect the regulation of CSE expression. Exogenous H_2S (10–80 μM) downregulates CSE transcription and expression in mammalian cells. The duration of action potential in healthy papillary muscles can be reduced by exogenous H_2S (50, 100, and 200 μM), and pretreatment with glibenclamide partially blocks the effects of 100 μM exogenous H_2S [51]. Exogenous H_2S at 120 μM significantly increases CSE transcription and expression [52]. Sodium hydrosulfide (NaHS; an inorganic H_2S

donor) and L-cysteine (L-Cys; a substrate of H_2S producing enzymes) decrease metabolic and vascular alterations induced by insulin resistance by reducing oxidative stress and activating endothelial nitric oxide synthase (eNOS) [53]. To a certain extent, CSE expression can be upregulated by hypoxia to a certain extent [30]. Supplementation with exogenous H_2S may inhibit FA-induced lung injury [54]. H_2S attenuates primary osteoporosis by preventing ROS-induced endoplasmic reticulum protein of 57 kDa (ERp57) damage in intestinal epithelial cells by enhancing ERp57 activity and promoting intestinal calcium absorption, thereby aiding in the development of therapeutic interventions to prevent osteoporosis [55]. Therefore, understanding the regulation of CSE expression in vascular endothelial cells under hypoxic conditions induced by exogenous H_2S is essential. Exogenous H_2S protects cytotrophoblasts against ceramide-induced necroptosis via the p38MAPK pathway [56]. Therefore, we studied the effect of 100 μM exogenous H_2S on CSE expression in human umbilical vein endothelial cells (HUVECs) under hypoxic conditions.

2. Materials and Methods

2.1. Cell Culture and Exogenous H₂S Treatment

The 293T cell line (Cat. GNHu17) were obtained from the Cell Bank of the Chinese Academy of Sciences, and human umbilical vein endothelial cells (HUVECs) were acquired from the School of Pharmacy at Fudan University. Abbreviations for technical terms were fully defined upon their first mention. The cultured cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Fisher Scientific International Inc.), 100 U/mL penicillin, and 0.1 mg/mL of streptomycin. The cells were exposed to exogenous hydrogen sulfide (H_2S) at a concentration of 100 μM H_2S (sodium hydrosulfide, NaHS) under hypoxic conditions at 37 °C for 0, 1, 2, 4, and 6 h. The control group received an equivalent volume of saline as the H_2S-treated group.

2.2. A reporter plasmid was constructed under the control of the CSE promoter

Human umbilical vein endothelial cells (HUVECs) were cultured until they achieved 80-90% confluence, at which point they were subjected to trypsin digestion and collected at 5000 rpm. Abbreviations for technical terms were defined upon their first use. The upstream and downstream primers for the cystathionine gamma-lyase (CSE) gene promoter sequence were designed to target a DNA fragment of 710 base pairs (bp), spanning from -696 to +16 nucleotides (nt). The researchers utilized pGL4.12-HuCSE710 as a template to amplify the 710-bp region upstream of the CSE gene using polymerase chain reaction (PCR), with the forward primer

5'- CGGGGTACCCATTAGGGGGAGTTTCTCTCTGT - 3' and the reverse primer 5' - CCGCTCGAGCTGCAGTCTCACGAT-CACAGT - 3'. The PCR product was digested with KpnI and XhoI restriction enzymes (Takara, China) and subsequently cloned into the pGL4.12 vector, which lacks a promoter (Promega, USA). Initially, the technical term abbreviations were defined. To construct the reporter plasmid containing the mutant CSE promoter, a similar methodology was employed, except for the use of an alternative forward primer (5'-CGGGGTACCCATTAGGATCT-GTTTCTCTCTGT-3').

2.3. Luciferase assay

HEK-293T cells were cultured to reach 70-80% confluence for transfection. The plasmids pGL4.12-HuCSE710 or pGL4.12-HuCSE710m, along with the pRL-CMV control vector, were introduced into the cells in 3.5 cm dishes using the Xfect™ Transfection Reagent (Takara Bio, Inc., USA). The activities of firefly and Renilla luciferases were measured 48 h post-DNA transfection.

3.Quantitative reverse transcription PCR (qRT-PCR) was performed

Total RNA was extracted using TransZol Up reagent (TransGen Biotech, China) after washing the treated cells twice with 1× dPBS. A LightCycler 96 System (Roche Molecular Systems, Inc.) was used for all reactions, utilizing a fluorescence quantification system. The QCSE forward and reverse primers (Table 1) were designed and used to assess the relative expression of CSE. After confirming that both ACTB (beta-actin gene) and CSE mRNA primers exhibited similar amplification efficiencies, the comparative Ct method, $2^{-\Delta\Delta CT}$, was applied to conduct the relative quantification of mRNA expression levels [57]. For western blotting, cells were incubated in PIPA lysis buffer on ice for 30 min. Subsequently, the lysates were centrifuged at 12,000 × g for 15 min at 4 °C. Proteins were then separated via electrophoresis on a 10% sodium dodecyl sulfate (SDS)-sulfate-polyacrylamide gel (Sangon Biotech, Shanghai, China) and subsequently transferred onto PVDF membranes (0.45 μm, Millipore, USA) to evaluate the expression of CSE and ACTB. The membranes were incubated with anti-CSE or anti-ACTB mouse monoclonal antibodies (Sangon Biotech Co., Ltd., Shanghai, China) at 4 °C for 12 h. An anti-mouse antibody (Sangon Biotech, Shanghai, China) was used to incubate the membrane. Positive CSE or ACTB bands were detected using BeyoECL Plus chemiluminescent substrate (Beyotime Biotechnology, China) at approximately 43-47 or 42-43 kDa, respectively.

Gene	GenBank Accession number	Forward Primer/Reversed Primer	Exon	Amplicon size
CSE	NM_001902.5	5'- GGCTCTACCTGCGTGCTTTA -3'	1	118 bp
		5'- CGCGAAAGAAGAAGAGAGGA-3'	1	

ACTB	NM_001101.3	5'- CTCTTCCAGCCTTCCTTCCT-3'	2	109 bp
		5'- TGTTGGCGTACAGGTCTTTG-3'	2	

Table 1: Primers Used for Quantitative Real-time PCR assays

3.1. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) derived from a minimum of four experimental replicates. Differences of statistical significance were assessed using a one-way analysis of variance (ANOVA) for repeated measures, followed by Tukey's post-hoc test. A P-value of less than 0.05 was considered statistically significant.

4. Results

4.1. Effect of Exogenous H_2S Administration on CSE Promoter Function During Oxygen Deprivation

This study investigated the impact of exogenous H_2S on CSE promoter activity under hypoxic conditions. As illustrated in Figure 1, HEK-293T cells were transfected and subjected to hypoxia for varying durations and subsequently divided into groups treated with 0 and 100 μM H_2S. In the absence of H_2S, promoter activity increased at 4 and 6 h compared to 1 and 2 h. Conversely, in the presence of 100 μM H_2S, the wild-type CSE promoter activity initially decreased at 1 and 2 h relative to the control but showed a slight increase at 4 and 6 h. For cells transfected and exposed to hypoxia for 1 and 2 h, the mutated CSE promoter activity in the 0 μM H_2S group decreased by 60-75% compared to the control. However, this activity increased at 4 and 6 h compared to the earlier time points. In the 100 μM H_2S group, mutated CSE promoter activity initially decreased at 1 and 2 h but demonstrated a slight increase at 4 and 6 h compared to the control. These findings suggest that exogenous H_2S modulates CSE promoter activity in HUVECs under hypoxic conditions.
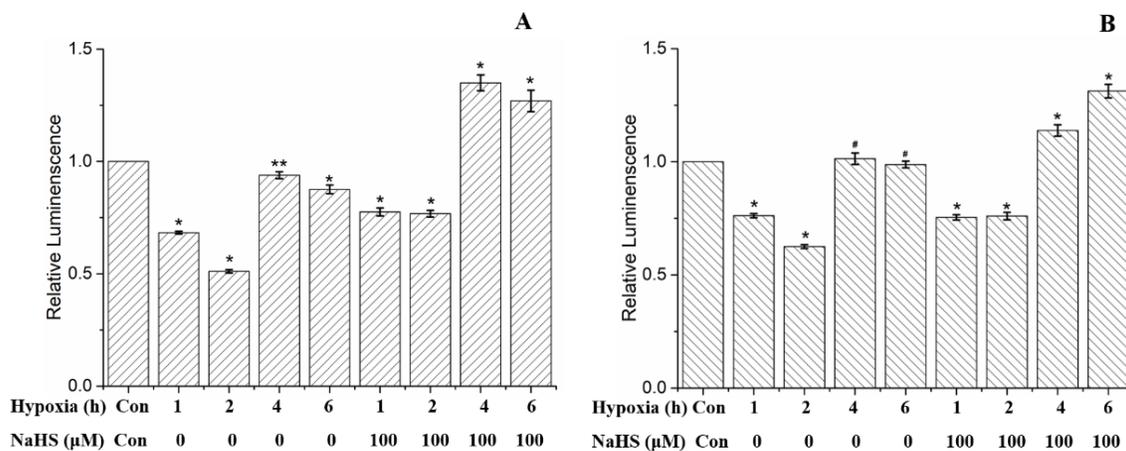

Figure 1: Impact of Exogenous H_2S on CSE Promoter Activity under Hypoxic Conditions

HEK-293T cells were subjected to hypoxia for 1, 2, 4, and 6-hours post-transfection and subsequently categorized into two groups: one without H_2S and the other with 100 μM H_2S. In the absence of H_2S, the activity of the wild-type CSE promoter was reduced by 50-70% at all time points compared to the control. For the mutated CSE promoter in the same group, activity decreased to 60-75% at 1 and 2 h relative to the control, followed by an increase in activity at 4 and 6 h. The mutated CSE promoter activity in the 0 μM H_2S group also declined at 1 and 2 h compared to the control group, with a slight increase observed at 4 and 6 h relative to the control group (*p<0.01; **p<0.05; # p>0.05).

4.2. Effect of Exogenous H_2S on CSE mRNA Expression under Hypoxic Conditions

This study examined the impact of exogenous hydrogen sulfide (H_2S) on cystathionine gamma-lyase (CSE) transcription under hypoxic conditions by assessing CSE mRNA expression in human umbilical vein endothelial cells (HUVECs). Figure 2 shows the results for HUVECs exposed to hypoxic conditions for 1, 2, 4, or 6 h. The experimental setup involved two groups: one without H_2S (0 μM) and the other with 100 μM H_2S. In the absence of H_2S, CSE mRNA expression in HUVECs showed a modest increase at 1, 2, and 4 h compared to the control group, followed by a decrease at 6 h relative to earlier time points. Conversely, exposure to 100 μM H_2S resulted in an initial decrease in CSE mRNA expression at 2 h compared to the control, but an increase was observed at 1, 4, and 6 h post-exposure. These results indicate that the presence of 100 μM H_2S modulates CSE mRNA expression in HUVECs, leading to a reduction compared to the conditions without H_2S.
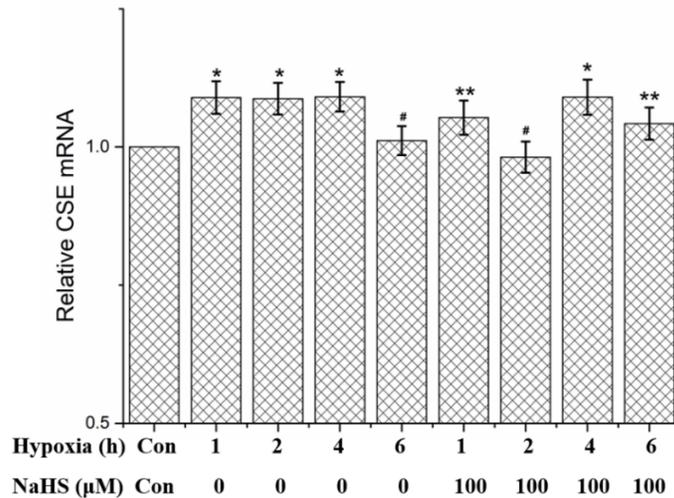
Figure 2: Impact of Exogenous H_2S on CSE mRNA Expression under Hypoxic Conditions

Human umbilical vein endothelial cells (HUVECs) were exposed to hypoxia for 1, 2, 4, and 6 h and subsequently categorized into two groups: 0 μM H_2S and 100 μM H_2S. In the absence of H_2S, CSE mRNA levels in HUVECs exhibited a modest increase at 1, 2, and 4 h compared to the control group. However, at 6 h, the expression decreased relative to earlier time points. Upon treatment with 100 μM H_2S, CSE mRNA expression was reduced at 2 h compared to that in the control. Conversely, at 1, 4, and 6 hours, CSE mRNA levels were elevated compared to the control group ($p<0.01$ for *, $p<0.05$ for **, and $p>0.05$ for # compared to the control).

4.3. Effect of Exogenous H_2S on CSE Expression under Hypoxic Conditions

This study investigated the impact of exogenous H_2S on CSE expression in HUVECs under hypoxic conditions. As illustrated in Figure 3, HUVECs were exposed to hypoxia for 1, 2, 4, and 6 h and subsequently categorized into groups receiving 0 and 100 μM H_2S treatment. In the absence of H_2S, CSE expression in HUVECs increased approximately two-fold at the 2-hour mark compared to that in the control. However, no significant alterations in CSE protein levels were detected at 1, 4, or 6 h relative to the control. Upon administration of 100 μM H_2S, CSE expression increased by approximately 50% at 1 and 2 h compared to the control but exhibited a slight decrease at 4 and 6 h. These findings suggest that 100 μM H_2S attenuated CSE expression in HUVECs following 2 h of hypoxia compared to the 0 μM H_2S condition.
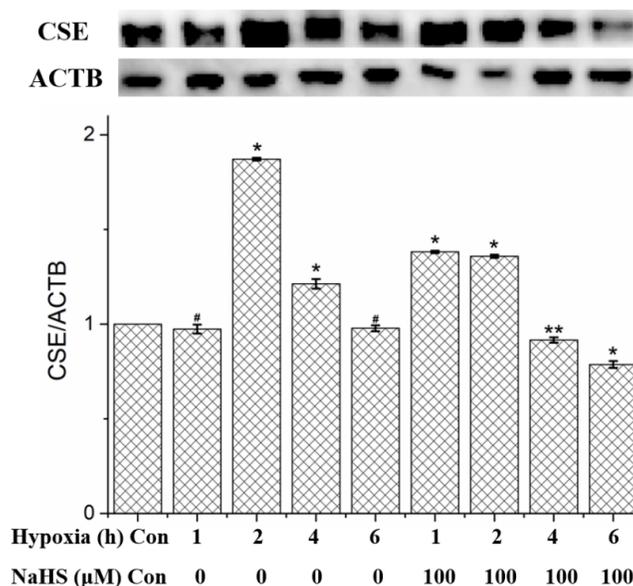

Figure 3: Effect of Exogenous H_2S on CSE Expression Under Hypoxic Conditions

In the 0 μM H_2S group, CSE protein expression in HUVECs exhibited an approximately two-fold increase at 2 h compared to the control group. However, CSE protein expression at 1, 4, and 6 h did not demonstrate significant changes relative to the control group. In the 100 μM H_2S group, the protein expression of CSE increased by approximately 50% at 1 and 2 h compared to the control group, whereas the protein expression of CSE at 4 and 6 h slightly decreased relative to the control group (*$p<0.01$; **$p<0.05$; #$p>0.05$, compared to the control group).

5. Discussion

To the best of our knowledge, this is the first study to examine the effect of exogenous H_2S on CSE expression in HUVECs under hypoxic conditions. Researchers have demonstrated that 100 μM exogenous H_2S regulates CSE expression in HUVECs under hypoxic conditions. Exogenous H_2S affects the transcriptional activity of *CSE* in mammalian cells [30]. As free H_2S is maintained at a low concentration under basal conditions, *CSE* mainly regulates its expression through feedback inhibition in the presence of low levels of exogenous H_2S (10–80 μM). However, exogenous H_2S (100 μM) inhibits the proliferation of HEK-293 cells [58]. Exogenous H_2S can inhibit the increase in pulmonary arterial pressure and decrease pulmonary vascular structural remodelling during hypoxic pulmonary hypertension (HPH) [59]. Longchamp et al. identified the requirement for CSE in vascular endothelial growth factor (VEGF)-dependent angiogenesis via increased H_2S production [60].

The CSE/H_2S pathway is indirectly associated with hypoxia, and H_2S protects mammalian cells from hypoxia-induced damage. Hypoxia causes apoptosis, which may play an essential role in ischemic heart disease, and increased tissue H_2S content protects the heart from ischemia/reperfusion injury [61,62]. Exogenous H_2S did not significantly alter the activity of mutated and wild-type CSE promoters under hypoxic conditions. Nevertheless, under hypoxic conditions, CSE promoter activity is influenced by exogenous H_2S. When exposed to 100 μM H_2S, as opposed to 0 μM H_2S, HUVECs exhibited decreased CSE mRNA and protein expression after two hours of hypoxia. CSE expression in HUVECs may be responsive to 100 μM exogenous H_2S under hypoxic conditions, and exogenous H_2S may modulate CSE expression over various time periods (1–6 h) under hypoxia.

Compared to the control groups, the introduction of exogenous H_2S resulted in a decrease in CSE expression after 2 h of hypoxic conditions. Moreover, exogenous H_2S reduced CSE expression in HUVECs under hypoxic conditions at various time points. These findings suggest that endothelial cells in blood vessels may respond to alterations in H_2S levels in the blood during periods of oxygen deprivation.

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