 doi:10.20944/preprints202010.0307.v1

Title

Exogenous H_2S Regulates of Cystathionine Gamma-Lyase in HUVECs during Hypoxia

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Short Title: H_2S Regulates CSE in HUVECs during Hypoxia

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Abstract

Cystathionine gamma-lyase (CSE) is one of the essential H_2S-producing enzymes, and it regulates diverse functions in connection with cardiovascular function. It is crucial how exogenous H_2S regulates CSE expression of the vascular endothelial cell during hypoxia. We examined the transcription and expression of *CSE* in HUVECs regulated by exogenous H_2S with 100 μM during hypoxia by Luciferase assay, Western blotting, and quantitative RT-qPCR. Exogenous H_2S influenced on the promoter activity of CSE in HUVECs during hypoxia. The effects of 100 μM H_2S on CSE mRNA expression in HUVECs is decreased compared with 0 μM H_2S. The consequences of 100 μM H_2S on the expression level of *CSE* protein in HUVECs at two h of hypoxia is reduced compared with 0 μM H_2S. These findings suggest that vascular endothelial cells can respond to the signals of hypoxia in the blood, and can respond to changes in H_2S concentration in the blood, thus affect the blood vessels themselves.

Keywords Cystathionine gamma-lyase; Hydrogen sulfide; Hypoxia; HUVECs

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Introduction

The endogenous generation of H_2S is mainly mediated by the enzyme cystathionine-γ-lyase (CSE) in the cardiovascular system[1]. Hydrogen sulfide (H_2S) has regarded as a signaling molecule as well as a cytoprotectant, and protects various tissues and organs from oxidative stress and ischemia-reperfusion injury[2]. Endothelial CSE contributes to cardiovascular homeostasis, primarily through the production of H_2S[3]. H_2S is produced in the vasculature and involved in promoting vascular homeostasis, vasodilation, and endothelial cell proliferation[4]. The vascular smooth muscle cells (SMCs) from the CSE gene knockout mice are more susceptible to apoptosis induced by exogenous H_2S at the physiologically relevant concentration[5]. The mechanisms of High level of homocysteine-induced endothelial dysfunction and the metabolism and physiological functions of H_2S as a protective agent[6]. After inhibiting endogenous background *CSE* expression, direct administration of exogenous H_2S at 100 μM can induce apoptosis of human aorta smooth muscle cells[7]. The mice over-expressed *CSE* in the heart have resistance to the ischemia-reperfusion injury, and the protection accompanied by a decrement in myocardial inflammation[8]. Endogenous H_2S plays modulatory roles in hypoxia-induced cardiovascular responses, inhibiting the cardiovascular in spontaneously hypertensive rats (SHR)[9]. The bath application of 100 μM exogenous H_2S can reduce the time required for the repolarization of the action potential [10].

Several studies have investigated the effects of H_2S in human vessels. H_2S-induced relaxation has demonstrated in internal mammary[11], pulmonary[12], mesenteric[13], and intrarenal arteries[14], as well as in perfused human placentas[15]. Up-regulation of CSE expression during hypoxia may increase the production and concentration of H_2S in cells and protecting cells from hypoxia[16]. A controlled release formulation of S-propargyl-cysteine showed protective effects

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against myocardial infarction (MI) via the CSE/H_2S pathway [17]. NADPH Oxidase 4 is a positive transcriptional regulator of CSE in endothelial cells and proposes that it may modulate the production of endogenous H_2S [18]. The duration of the action potential in the healthy papillary muscles can cut down by exogenous H_2S (50, 100, 200 μM), and pretreatment with glibenclamide partly blocks the effects of exogenous H_2S at 100 μM[19].

It is crucial how exogenous H_2S regulates CSE expression of the vascular endothelial cell during hypoxia. Therefore, we study the effects of exogenous H_2S on *CSE* expression in HUVECs during hypoxia.

Materials and Methods

It was the construction of the reporter under the CSE promoter. HUVECs were cultured to a confluence of 80-90%, digested with trypsin, and collected at 5000 r/min. It was extracting the genome DNA of HUVECs by using 1% agarose gel. Searching the sequence of CSE gene promoter in the GenBank database, designing upstream and downstream primers, and the target fragment DNA length was 710bp (-696~+16nt). According to the CSE (NC_000001.11), we amplified the 710 bp DNA upstream of the CSE gene by PCR using pGL4.12-HuCSE710 as the template (forward primer 5'-CGGGGTACCCATTAGGGGGAGTTTCTCTCTGT-3' and reverse primer 5'- CCGCTCGAGCTGCAGTCTCACGATCACAGT -3'). The thermal cycling condition as follows: initial denaturation at 94 °C/3 min; followed by 30 cycles with 95 °C/30 sec, 60 °C/45 sec, 72 °C/1 min 30 sec; final step at 72 °C/10 min. To digest the PCR product with the restriction enzymes Kpn I and Xho I (TAKARA, China) and cloned into the promoterless pGL4.12 (Promega, USA). To designating the resultant construct as pGL4.12-HuCSE710, and confirming the inserted DNA fragment by DNA sequencing of Sangon Biotech (Shanghai) Co.,

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Ltd. Construction of the reporter of the mutant CSE promoter except that an alternative forward primer (5'-CGGGGTACCCATTAGGATCTGTTTCTCTCTGT-3') which used during PCR amplification. The fragment size was identified and sequenced by Sangon Biotech (Shanghai) Co., Ltd.

Cell culture and treatments. We purchased HEK-293T cell lines and HUVECs from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cultured cells maintained in DMEM supplemented with 10% Fetal bovine serum (FBS, Fisher Scientific International Inc.), 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humidified atmosphere composed of 95% air and 5% CO_2 at 37°C. For treatment with exogenous H_2S, cells incubated with 100 μM H_2S at 37°C during hypoxia for 0 h, one h, two h, four h, and six h. After incubation and removing the cell medium, Luciferase assay, quantitative real-time PCR, and western blotting were carried out as described below.

Luciferase assay. For transfection, HEK-293T cells were grown to 70-80% confluent. The 5 μg pGL4.12-HuCSE710 or 5 μg pGL4.12-HuCSE710m together with 0.028μg of the pRL-CMV control vector has transected the cells per 3.5 dish using Xfect™ transfection reagent (Takara Bio USA, Inc). After 12 h, the transfected cells were sub-cultured in several at the proportion of 1:3 for 24 h. After six h, we measured the firefly and Renilla luciferase activities after 48 h of DNA transfection.

Quantitative real-time PCR. The total RNA isolated using the *TransZol* Up reagent (TransGen Biotech, China) after the treated cells rinsed with 1× dPBS buffer twice. Dissolve the extracted RNA in RNase-free water bypassing the solution a few times through a pipette tip, incubating for 10 min at 55 to 60 °C and cooling to Room temperature. To synthesize the first-strand cDNA at 42 °C for 30 min using anchored oligo$^{(dT)}$18 primer. The reaction mixture was in a total volume

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of 20 µL containing 2 µg of RNA, one µL of anchored oligo$^{(dT)}$18 primers, ten µL of 2× TS Reaction Mix and *TransScript*^{TM} RT/RI Enzyme mix (TransGen Biotech, China) as well as RNase-free water. To perform Quantitative Real-time PCR in a final volume of 25 µL (TransGen Biotech, Beijing, China). All reactions run with the LightCycler® 96 System (Roche Molecular Systems, Inc.) using a fluorescence quantification system with the following conditions: the conditions: an initial step at 95 °C/ten min, 45 cycles with 30 sec/ 95 °C, 30 sec/60 °C, and ten sec/72 °C. To design the primer pair Q *CSE* Forward Primer / Q *CSE* Reversed Primer (Table 1) to determine the relative expression of *CSE*. The primers specific to PCR templates carried out by the online software http://www.ncbi.nlm.nih.gov/tools/primer-blast/. To measure the fluorescence at the end of the extension step at 72 °C. Controls for genomic DNA and primer contamination routinely performed with non-RT or no template PCR reactions, respectively. To show dissociation curves for each set of oligonucleotides to check primer specificity and to confirm the presence of a unique PCR product. Standard curves performed based on five serial dilutions of the cDNA stock. PCR efficiency of the primer sets was between 95 and 100 %. By verifying both *ACTB* (beta-Actin gene) and *CSE* mRNA primers had similar amplifying efficiency, we use the comparative Ct method $2^{-\Delta\Delta}$Ct for performing relative quantification analysis of mRNA levels[20]. The relative amount of each mRNA of the control one defined as 1.0. To estimate the number of transcripts from a standard line derived from 20-fold serial dilutions of cDNA pooled from HUVECs treated with LPS.

Table 1: Primers used for quantitative real-time PCR assays

Gene	GenBank Accession number	Forward Primer/Reversed Primer	Exon	Amplicon size
CSE	NM_001902.5	5'- GGCTCTACCTGCGTGCTTTA -3'	1	118bp
		5'- CGCGAAAGAAGAAGAGAGGA-3'	1	
ACTB	NM_001101.3	5'- CTCTTCCAGCCTTCCTTCCT-3'	2	109 bp

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		5'- TGTTGGCGTACAGGTCTTTG-3'	2	

Western Blot. For total protein extraction, to incubate 0.5×10^6 HUVECs in 120 μL of PIPA lysis buffer (mild) (TransGen Biotech, China), supplemented with one mM PMSF proteinase, 0.25 U/μL Benzonase, inhibitor cocktail (Takara Bio USA, Inc). To incubate both cells and PIPA lysis buffer on ice for 30 min, and the lysate was centrifugated at 12 000 × g at 4 °C for 15 min. The protein was separated by electrophoresis on 10 % for detecting to CSE. ACTB sodium dodecyl sulfate (SDS)-polyacrylamide gel (Sangon Biotech, Shanghai) and transferred onto PVDF membrane (0.45 μM, Millipore, USA). The membrane was incubated at 4°C with Anti-CSE (Sangon Biotech Co., Ltd. Shanghai) mouse monoclonal antibodies (1:1, 000 dilutions) or anti-ACTB (Sangon Biotech Co., Ltd. Shanghai) mouse monoclonal antibodies (1:2, 000 dilutions) for 12 hr. We incubated the membrane with an anti-mouse antibody (1:5, 000) (Sangon Biotech, Shanghai). Positive bands for CSE or ACTB were identified around 43-47, or 42-43 kDa, respectively, by BeyoECL Plus chemiluminescent substrate (Beyotime Biotechnology, China). The results were scanned and quantified using FluorChem HD2 sensitive chemiluminescent imaging with its software (ProteinSimple, USA).

Statistical analysis. All data expressed with mean ± SEM of at least four experiments. A statistical significance assesses with either one-way ANOVA or two-way ANOVA for repeated measures followed by Turkey's test. $P < 0.05$ was considered significant.

Results

Effect of exogenous H_2S on *CSE* promoter activity during hypoxia. We analyzed the effects of exogenous H_2S on the activities of the CSE promoter during hypoxia, as showed in Figure 1A. The transfected HEK-293T was induced by hypoxia for one h, two h, four h, six h, and divide

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these transfected cells into the 0 μM H_2S group and the 100 μM H_2S group. In the 0 μM H_2S group, the CSE wild promoter activity decreased to 50-70% at one h, two h, four h, six h of the 0 μM H_2S group compared to control. However, the promoter activity of at four h and six h increase recoverable compared to one h, and two h; in the 100 μM H_2S group, the CSE wild promoter activity decreased at one h, two h, of the 100 μM H_2S group compared to control. Still, the promoter activity of at four h and six h increased slightly compared to control, as shown in Fig. 1B, the transfected HEK-293T was induced by hypoxia for one h, two h, and these transfected cells were divided into the 0 μM H_2S group and the 100 μM H_2S group. In the 0 μM H_2S group, the CSE mutation promoter activity decreased to 60-75% at one h, two h of the 0 μM H_2S group compared to control, but the promoter activity of at four h and six h increase recoverable compared to 1 h and two h. In the 0 μM H_2S group, the CSE mutation promoter activity decreased at one h, two h of the 100 μM H_2S group compared to control, but the promoter activity at four h and six h increased slightly compared to control. Exogenous H_2S influenced on the promoter activity of CSE in HUVECs during hypoxia.

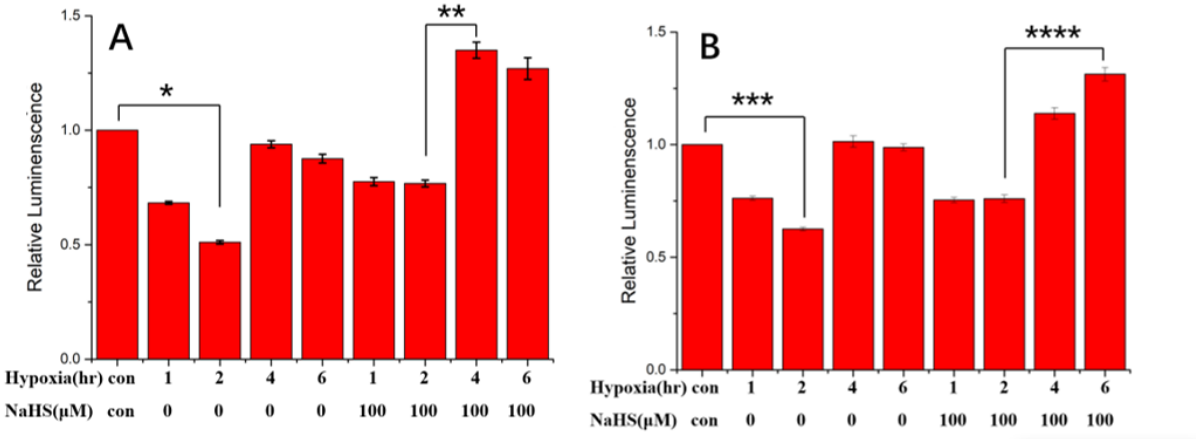

Figure 1: Effect of exogenous H S on the CSE promoter activity during hypoxia. There were no remarkable changes between the effects of exogenous H_2S on the CSE mutation promoter

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activity and the CSE wild the transfected HEK-293T cells during hypoxia. (*P<0.05: two h of 0 μM H_2S group vs con; **P<0.05: four h vs one h and two h in 0 μM H_2S group; ***P<0.01: two h of 100 μM H_2S vs control; ****P>0.05: two h vs six h of 100 μM H_2S)

Effect of exogenous H_2S on *CSE* at the mRNA level during hypoxia. To analyze the effects of exogenous H_2S on CSE transcription during hypoxia, we examined the CSE mRNA expression in HUVECs. As showed in Fig. 2, HUVECs were induced by hypoxia for one h, two h, four h, and six h; divide HUVECs into the 0 μM H_2S group and the 100 μM H_2S group. In the 0 μM H_2S group, the CSE mRNA expression in HUVECs increased slightly at one h, two h, four h of the 0 μM H_2S group compared to control. However, the CSE mRNA expression of at six h decreased compared to 1 h, two h, and four h. In the 100 μM H_2S group, the CSE mRNA expression decreased at two h of the 100 μM H_2S group compared to control. However, the CSE mRNA expression at one h, four h, and six h increased compared to control. The result shows that the effects of 100 μM H_2S on CSE mRNA expression in HUVECs is decreased compared with 0 μM H_2S.

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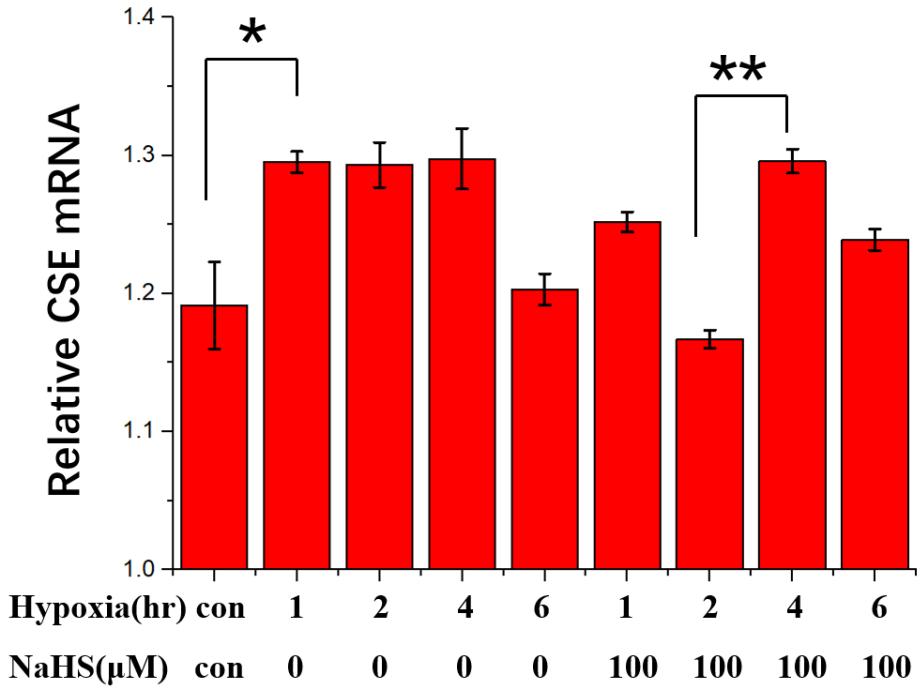

Figure 2: Effect of exogenous H_2S on CSE at the mRNA level during hypoxia. The effects of 100 μM H_2S on CSE mRNA expression in HUVECs is decreased compared with 0 μM H_2S. (*P<0.05: one h of 0 μM H_2S group vs con; **p<0.01: two h vs four h of 100 μM H_2S)

Effect of exogenous H_2S on *CSE* at the protein level during hypoxia. We also examined the effects of exogenous H_2S on the expression level of *CSE* protein in HUVECs during hypoxia. As showed in Fig. 3, HUVECs were induced by hypoxia for one h, 2h, four h, and six h, and divide HUVECs into the 0 μM H_2S group and the 100 μM H_2S group. In the 0 μM H_2S group, the expression level of *CSE* protein in HUVECs increased almost double at two h compared to control. Still, the expression level of *CSE* protein at one h, four h, and six h did not change remarkably compared to control. In the 100 μM H_2S group, the expression level of *CSE* protein increased to about 50% at one h, two h compared to control, but the expression level of *CSE* protein at four h and six h decreased a few compared to control. The result shows that the effects

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of 100 μM H_2S on the expression level of *CSE* protein in HUVECs at two h of hypoxia is decreased compared with 0 μM H_2S.

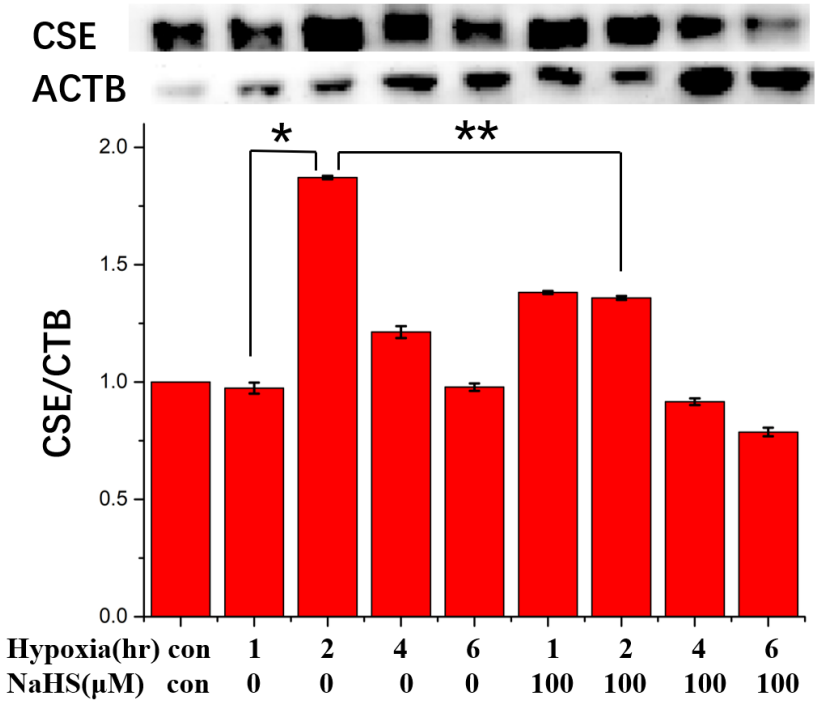

Figure 3: Effect of exogenous H_2S on *CSE* at the protein level during hypoxia. The effects of 100 μM H_2S on the expression level of CSE protein in HUVECs at two h of hypoxia is decreased compared with 0 μM H_2S. (*P<0.05: *P<0.01: two h of 100 μM H_2S group vs two h of 0 μM H_2S group)

Discussion

In this experiment, we first investigated the regulatory mechanism of exogenous H_2S on *CSE* in HUVECs during hypoxia. We demonstrated that exogenous H_2S of 100 μM is involved in the regulation of *CSE* expression in HUVECs during hypoxia. Exogenous H_2S could affect the transcriptional activity of mouse *CSE* in mammalian cells[16]. As the level of free H_2S is maintained at a low concentration under basal conditions[21], so *CSE* mainly adjusts itself through the *CSE* feedback inhibition at the lower level of exogenous H_2S (from 10 to 80 μM).

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However, an exogenous H_2S (100μM) can inhibit the proliferation of HEK-293 cells [22]. Exogenous H_2S could oppose the elevation of pulmonary arterial pressure and lessen the pulmonary vascular structure remodeling during hypoxic pulmonary hypertension (HPH)[23]. The Longchamp's group identified a requirement for CSE in vascular endothelial growth factor (VEGF) dependent angiogenesis via increased H_2S production[24].

The CSE/H_2S pathway has indirectly linked to hypoxia, and H_2S can protect mammalian cells against hypoxia-induced injuries. Hypoxia causes apoptosis, which may play essential roles in ischemic heart disease[25], and increased tissue content of H_2S protects the heart from ischemia/reperfusion damage[26]. There is no significant difference between the effects of exogenous H_2S on the CSE mutation promoter activity and the CSE wild during hypoxia. However, exogenous H_2S influenced on the promoter activity of CSE. The effects of 100 μM H_2S on CSE mRNA expression in HUVECs is decreased compared with 0 μM H_2S. The consequences of 100 μM H_2S on the expression level of *CSE* protein in HUVECs at two h of hypoxia is reduced compared with 0 μM H_2S. Undoubtedly, *CSE* expression in HUVECs can respond to exogenous H_2S of 100 μM during hypoxia, and *CSE* expression in HUVECs is regulated by exogenous H_2S during hypoxia with different time of from 1 h to 6 h.

All in all, compared with the control group, exogenous H_2S can down-regulate the expression of CSE gene under hypoxia during hypoxia for two h. Exogenous H_2S can affect and down-regulate the expression of CSE gene in HUVECs during hypoxia at other times. These findings suggest that vascular endothelial cells can respond to the signals of hypoxia in the blood, and can respond to changes in H_2S concentration in the blood, thus affect the blood vessels themselves.

Acknowledgements

Natural Science Foundation of Guangdong Province (Grant No. 2016A030307039) supported the project.

Conflict of Interest

The author declares that they have no competing interests.

Ethics and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors. In this study, we have not collected any samples of humans and animals.

Data Availability Statement

The data of this study are available from the corresponding author upon reasonable request.

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