Exogenous H$_2$S regulates the expression of cystathionine gamma-lyase in HUVECs during hypoxia

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Short Title: H$_2$S Regulates CSE in HUVECs during Hypoxia

Abstract

Cystathionine gamma-lyase (CSE) is an essential Hydrogen sulphide (H_2S)-producing enzyme that regulates diverse processes related to cardiovascular function. It is crucial to understand how exogenous H_2S regulates *CSE* expression in vascular endothelial cells during hypoxia. We examined the regulatory effect of 100 μM H_2S on the transcription and expression of *CSE* in HUVECs during hypoxia by luciferase assay, Western blotting, and quantitative RT-qPCR. Exogenous H_2S influenced the promoter activity of CSE in HUVECs during hypoxia. Compared with 0 μM H_2S, 100 μM H_2S decreased the mRNA expression of *CSE* in HUVECs. Compared with 0 μM H_2S, 100 μM H_2S decreased the protein expression of *CSE* in HUVECs after 2 h of hypoxia. These findings suggest that vascular endothelial cells can respond to changes in H_2S concentration in the blood during hypoxia.

Keywords: Cystathionine gamma-lyase; Hydrogen sulphide; Hypoxia; HUVECs

Introduction

The endogenous generation of hydrogen sulphide (H_2S) is mainly mediated by the enzyme cystathionine-γ-lyase (CSE) in the cardiovascular system[1]. H_2S is considered a signalling molecule and a cytoprotectant that protects various tissues and organs from oxidative stress and ischaemia-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 promotes vascular homeostasis, vasodilation, and endothelial cell proliferation[4]. 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[5]. High levels of homocysteine induce endothelial cell dysfunction, and the metabolism and physiological functions of H_2S allow it to function a protective agent[6]. After inhibiting endogenous background CSE expression, the direct administration of 100 μM exogenous H_2S can induce the apoptosis of human aorta smooth muscle cells[7]. Mice overexpressing CSE in the heart exhibit resistance to ischaemia-reperfusion injury, and this protection is accompanied by a decrease in myocardial inflammation[8]. Endogenous H_2S plays modulatory roles in hypoxia-induced cardiovascular responses, inhibiting cardiovascular disease in spontaneously hypertensive rats (SH)[9].

Several studies have investigated the effects of H_2S in human vessels. H_2S-induced relaxation has been demonstrated in internal mammary[10], pulmonary[11], mesenteric[12], and intrarenal arteries[13] as well as in perfused human placentas[14]. CSE-derived H_2S production by endothelial cells is critical for maintaining endothelial function and exercise capacity and for protecting against myocardial ischaemia/reperfusion injury[15]. Upregulation of CSE expression during hypoxia may increase the production and concentration of H_2S in cells and protect cells

from hypoxia[16]. A controlled release formulation of S-propargyl-cysteine exerted protective effects 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 some researchers propose that it may modulate the production of endogenous H_2S [18].

An in vitro study showed that an exogenous H_2S donor attenuated hypoxia-induced apoptosis in primary rat nucleus pulposus (NP) cells [19]. H_2S has been identified as an excitatory mediator of hypoxic sensing in carotid bodies [20]. Incubation with sodium hydrosulfide (NaHS), an H_2S donor, increased the expression of miR-21 and attenuated the reduced cell viability and the increased apoptosis caused by ischaemia-reperfusion (I/R) in BRL cells[21]. The exogenous administration of NaHS might be a potential strategy for the treatment of nickel-induced lung cancer progression[22]. Pretreatment with NaHS or aspirin (ATB-340) in aged rats fed a high-fructose diet (HFD) and animals exposed to water immersion restraint stress (WIRS) attenuated gastric damage compared to vehicle treatment [23].

Some studies have shown that appropriate levels of exogenous H_2S can affect the regulation of CSE expression. Exogenous H_2S at 10-80 μM downregulates the transcription and expression of CSE in mammalian cells. Exogenous H_2S at 120 μM significantly increases the transcription and expression of CSE [24]. The duration of the action potential in the 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[25]. CSE expression can be upregulated by hypoxia to a certain extent[16]. It is crucial to understand how exogenous H_2S regulates CSE expression in vascular endothelial cells during hypoxia, so we studied the effects of 100 μM exogenous H_2S on CSE expression in HUVECs during hypoxia.

Materials and Methods

Cell culture and treatments. The 293T cell line (Cata. GNHu17) was purchased from the Cell Bank of the Chinese Academy of Sciences, and HUVECs were purchased from the School of Pharmacy of Fudan University. The cultured cells were maintained in DMEM supplemented with 10% foetal bovine serum (FBS, Fisher Scientific International Inc.), 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. For treatment with exogenous H_2S, the cells were incubated with 100 μM H_2S (NaHS, a donor of H_2S) at 37 °C under hypoxic conditions for 0 h, 1 h, 2 h, 4 h, and 6 h. After incubation and removal of the cell medium, luciferase assays, quantitative real-time PCR, and Western blotting were carried out as described below.

Construction of a reporter plasmid under the control of the CSE promoter. HUVECs were cultured to 80-90% confluence, digested with trypsin, and collected at 5000 r/min. Genomic DNA was extracted from the HUVECs by using a 1% agarose gel. The CSE gene promoter sequence was obtained by the GenBank database, upstream and downstream primers were designed, and the target fragment DNA length was 710 bp (-696~+16 nt). According to the CSE sequence (NC_000001.11), we amplified the 710-bp region 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 conditions were as follows: initial denaturation at 94 °C/3 min; 30 cycles of 95 °C/30 sec, 60 °C/45 sec, and 72 °C/1 min 30 sec; and a final step at 72 °C/10 min. The PCR product was digested with the restriction enzymes Kpn I and Xho I (Takara, China) and cloned into the promoterless vector pGL4.12 (Promega, USA). The resultant construct was designated pGL4.12-HuCSE710, and the inserted

DNA fragment was confirmed by DNA sequencing by Sangon Biotech Co., Ltd. (Shanghai). The reporter plasmid with the mutant CSE promoter was constructed following a similar protocol except that an alternative forward primer (5'-CGGGGTACCCATTAGGATCTGTTTCTCTCTGT-3') was used during the PCR amplification. The fragment size was identified and sequenced by Sangon Biotech Co., Ltd. (Shanghai).

Luciferase assay. For transfection, HEK-293T cells were grown to 70-80% confluence. A total of 5 μg pGL4.12-HuCSE710 or 5 μg pGL4.12-HuCSE710m together with 0.028 μg pRL-CMV control vector was transfected into cells in 3.5 cm dishes using Xfect™ transfection reagent (Takara Bio, Inc., USA). After 12 h, the transfected cells were subcultured at a proportion of 1:3 and cultured for an additional 24 h. After 6 h, we measured the firefly and Renilla luciferase activities 48 h after DNA transfection.

Quantitative real-time PCR. Total RNA was isolated using *TransZol* Up reagent (TransGen Biotech, China) after the treated cells were washed twice with $1\times$ dPBS buffer. The extracted RNA was dissolved in RNase-free water, passing the solution through a pipette tip a few times; then, the solution was incubated for 10 min at 55 to 60 °C and cooled to room temperature. First-strand cDNA was synthesized at 42 °C for 30 min using an anchored oligo$^{(dT)}$18 primer. The 20-μL reaction mixture contained 2 μg of RNA, 1 μL of anchored oligo$^{(dT)}$18 primers, 10 μL of $2\times$ TS Reaction Mix and *TransScript*^{TM} RT/RI Enzyme mix (TransGen Biotech, China) and RNase-free water. Quantitative real-time PCR was performed in a final volume of 25 μL (TransGen Biotech, Beijing, China). All the reactions were run with the LightCycler® 96 System (Roche Molecular Systems, Inc.) using a fluorescence quantification system with the following conditions: an initial step at 95 °C/10 min followed by 45 cycles of 30 sec/95 °C, 30 sec/60 °C, and 10 sec/72 °C. The Q *CSE* Forward Primer/Q *CSE* Reversed Primer (Table 1) primers were

designed and used to determine the relative expression of *CSE*. Primers specific to the cDNA templates were designed by the online software http://www.ncbi.nlm.nih.gov/tools/primer-blast/. The fluorescence was measured at the end of the extension step at 72 °C. Controls for genomic DNA and primer contamination were routinely performed with non-RT or no template reactions, respectively. Dissociation curves were generated for each set of oligonucleotides to check primer specificity and to confirm the presence of a unique PCR product. Standard curves generated based on five serial dilutions of the cDNA stock. The PCR efficiency of the primer sets was between 95 and 100%. Arter verifying that both the *ACTB* (beta-Actin gene) and *CSE* mRNA primers had similar amplification efficiencies, we used the comparative Ct method $2^{-\Delta\Delta C_T}$ to perform relative quantification analysis of the mRNA expression levels[26]. The relative amount of each mRNA in the control sample was defined as 1.0. The number of transcripts were estimated based on a standard curve derived from 20-fold serial dilutions of pooled cDNA from HUVECs treated with LPS.

Western blotting. For total protein extraction, 0.5×10^6 HUVECs were incubated with 120 µL of PIPA lysis buffer (mild) (TransGen Biotech, China) supplemented with 1 mM PMSF, and 0.25 U/µL Benzonase proteinase inhibitor cocktail (Takara Bio USA, Inc). The cells were incubated with PIPA lysis buffer on ice for 30 min, and the lysates were centrifuged at 12 000 × g at 4 °C for 15 min. The proteins were separated by electrophoresis on 10 % sodium dodecyl sulphate (SDS)-polyacrylamide gel (Sangon Biotech, Shanghai) and transferred onto PVDF membranes (0.45 µM, Millipore, USA) to detect CSE and ACTB expression. The membranes were incubated at 4 °C with anti-CSE mouse monoclonal antibodies (1:1,000 dilutions; Sangon Biotech Co., Ltd., Shanghai) or anti-ACTB mouse monoclonal antibodies (1:2,000 dilutions; Sangon Biotech Co., Ltd., Shanghai) for 12 h. We incubated the membranes with an anti-mouse antibody

(1:5,000) (Sangon Biotech, Shanghai). Positive CSE or ACTB bands were identified at approximately 43-47 or 42-43 kDa, respectively, by BeyoECL Plus chemiluminescent substrate (Beyotime Biotechnology, China). The results were imaged and quantified using FluorChem HD2-sensitive chemiluminescent imaging and its software (ProteinSimple, USA).

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

Results

Effect of exogenous H₂S on *CSE* promoter activity during hypoxia. We analysed the effects of exogenous H_2S on CSE promoter activity during hypoxia, and the results are shown in Figure 1. Transfected HEK-293T cells were exposed to hypoxic conditions for 1 h, 2 h, 4 h, and 6 h, and these transfected cells were divided into the 0 μM H_2S group and the 100 μM H_2S group. However, the promoter activity at 4 h and 6 h increased compared to that observed at 1 h and 2 h; in the 100 μM H_2S group, the wild-type CSE promoter activity decreased at 1 h and 2 h compared to that in the control group. Nevertheless, the promoter activity at 4 h and 6 h increased slightly compared to that of the control, as shown in Figure 1. The transfected HEK-293T cells were exposed to hypoxia for 1 h and 2 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 mutated CSE promoter activity decreased to 60-75% at 1 h and 2 h compared to that in the control, but the promoter activity at 4 h and 6 h increased compared to that observed at 1 h and 2 h. In the 0 μM H_2S group, the mutated CSE promoter activity decreased at 1 h and 2 h compared to that in the control group, but the promoter activity at 4 h and 6 h slightly increased compared to that in the

control group. Thus, exogenous H_2S influenced the promoter activity of CSE in HUVECs during hypoxia.

Effect of exogenous H_2S on *CSE* mRNA expression during hypoxia. To analyse the effects of exogenous H_2S on CSE transcription during hypoxia, we examined CSE mRNA expression in HUVECs. As shown in Figure 2, HUVECs were exposed to hypoxic conditions for 1 h, 2 h, 4 h, and 6 h, and the HUVECs 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 mRNA expression in HUVECs increased slightly at 1 h, 2 h, and 4 h compared to that in the control group. However, the CSE mRNA expression at 6 h decreased compared to that observed at 1 h, 2 h, and 4 h. In the 100 μM H_2S group, the CSE mRNA expression decreased at 2 h compared to that in the control group. However, the CSE mRNA expression at 1 h, 4 h, and 6 h increased compared to that in the control group. The results showed that 100 μM H_2S decreased CSE mRNA expression in HUVECs compared with 0 μM H_2S.

Effect of exogenous H_2S on *CSE* expression during hypoxia. We also examined the effects of exogenous H_2S on CSE expression in HUVECs during hypoxia. As shown in Figure 3, HUVECs were exposed to hypoxic conditions for 1 h, 2 h, four h, and 6 h, and then, the HUVECs were divided into the 0 μM H_2S group and the 100 μM H_2S group. In the 0 μM H_2S group, the expression of *CSE* in HUVECs almost doubled at 2 h compared to that in the control group. Nevertheless, the protein expression of *CSE* at 1 h, 4 h, and 6 h did not markedly change compared to that in the control group. In the 100 μM H_2S group, the expression of *CSE* increased to approximately 50% at 1 h and 2 h compared to that in the control group, but the protein expression of *CSE* at 4 h and 6 h decreased slightly compared to that in the control group. The

results showed that 100 μM H_2S decreased the expression of *CSE* in HUVECs after 2 h of hypoxia compared with 0 μM H_2S.

Discussion

This experiment first investigated the mechanism by which exogenous H_2S regulates *CSE* expression in HUVECs during hypoxia. We demonstrated that 100 μM exogenous H_2S participates in regulating *CSE* expression in HUVECs during hypoxia. Exogenous H_2S could affect the transcriptional activity of mouse *CSE* in mammalian cells[16]. As free H_2S is maintained at a low concentration under basal conditions, *CSE* mainly regulates its expression through *CSE* feedback inhibition in the presence of lower levels of exogenous H_2S (from 10 to 80 μM). However, exogenous H_2S (100 μM) can inhibit the proliferation of HEK-293 cells [27]. Exogenous H_2S can inhibit the increase pulmonary arterial pressure and decrease pulmonary vascular structure remodelling during hypoxic pulmonary hypertension (HPH)[28]. Longchamp's group identified a requirement for CSE in vascular endothelial growth factor (VEGF)-dependent angiogenesis via increased H_2S production[29].

The CSE/H_2S pathway is indirectly linked to hypoxia, and H_2S can protect mammalian cells against hypoxia-induced injury. Hypoxia causes apoptosis, which may play essential roles in ischaemic heart disease[30], and increased tissue content of H_2S protects the heart from ischaemia/reperfusion damage[31]. There was no significant difference between the effects of exogenous H_2S on mutated CSE promoter activity and CSE wild-type promoter activity during hypoxia. However, exogenous H_2S influenced the promoter activity of CSE. Compared with 0 μM H_2S, 100 μM H_2S decreased CSE mRNA expression in HUVECs. Compared with 0 μM H_2S, 100 μM H_2S decreased the protein expression of *CSE* in HUVECs after 2 h of hypoxia. Clearly, *CSE* expression in HUVECs can respond to 100 μM exogenous H_2S during hypoxia,

and *CSE* expression in HUVECs is regulated by exogenous H_2S during hypoxia at different times, from 1 h to 6 h.

Overall, compared with the control, exogenous H_2S downregulated the expression of the CSE gene during exposure to hypoxic conditions for 2 h. Exogenous H_2S affected and downregulated CSE gene expression in HUVECs during hypoxia at other time points. These findings suggest that vascular endothelial cells can respond to changes in H_2S concentrations in the blood during hypoxia.

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Conflict of Interest

The author declares that there are no competing interests.

Ethics and Consent to Participate

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

Data Availability Statement

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

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Tables

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	118 bp
		5'- CGCGAAAGAAGAAGAGAGGA-3'	1	
ACTB	NM_001101.3	5'- CTCTTCCAGCCTTCCTTCCT-3'	2	109 bp
		5'- TGTTGGCGTACAGGTCTTTG-3'	2	

Figure legends

Figure 1: Effect of exogenous H S on CSE promoter activity during hypoxia.

Transfected HEK-293T cells were exposed to hypoxia for 1 h, 2 h, 4 h, and 6 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 wild-type CSE promoter activity decreased to 50-70% at 1 h, 2 h, 4 h, and 6 h compared to that in the control group. In the 0 μM H_2S group, the mutated CSE promoter activity decreased to 60-75% at 1 h and 2 h compared to that in the control group, but the promoter activity at 4 h and 6 h increased compared to that observed at 1 h and 2 h. In the 0 μM H_2S group, the mutated CSE promoter activity decreased at 1 h, 2 h compared to that in the control group, but the promoter activity at 4 h and 6 h slightly increased compared to that in the control group (*p<0.01; **p<0.05; $^\#$ p>0.05 when compared to the control group).

Figure 2: Effect of exogenous H_2S on CSE mRNA expression during hypoxia.

HUVECs were exposed to hypoxic conditions for 1 h, 2 h, 4 h, and 6 h and then divided 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 slightly increased at 1 h, 2 h, and 4 h compared to that in the control group. However, the CSE mRNA expression at 6 h decreased compared to that observed at 1 h, 2 h, and 4 h. In the 100 μM H_2S group, the CSE mRNA expression decreased at 2 h compared to that in the control group. However, CSE mRNA expression at 1 h, 4 h, and 6 h increased compared to that in the control group (*p<0.01; **p<0.05; $^\#$p>0.05 when compared to the control group).

Figure 3: Effect of exogenous H_2S on *CSE expression* during hypoxia.

HUVECs were exposed to hypoxia for 1 h, 2 h, 4 h, and 6 h and divided into the 0 μM H_2S group and the 100 μM H_2S group. In the 0 μM H_2S group, the protein expression of *CSE* in HUVECs almost doubled at 2 h compared to that in the control group. Nevertheless, the protein expression of *CSE* at 1 h, 4 h, and 6 h did not markedly change compared to that in the control group. In the 100 μM H_2S group, the protein expression of *CSE* increased to approximately 50% at 1 h and 2 h compared to that in the control group,

but the protein expression of *CSE* at 4 h and 6 h slightly decreased compared to that in the control group (*p<0.01; **p<0.05; $^{#}$p>0.05 compared to the control group).

Figures

Figure 1

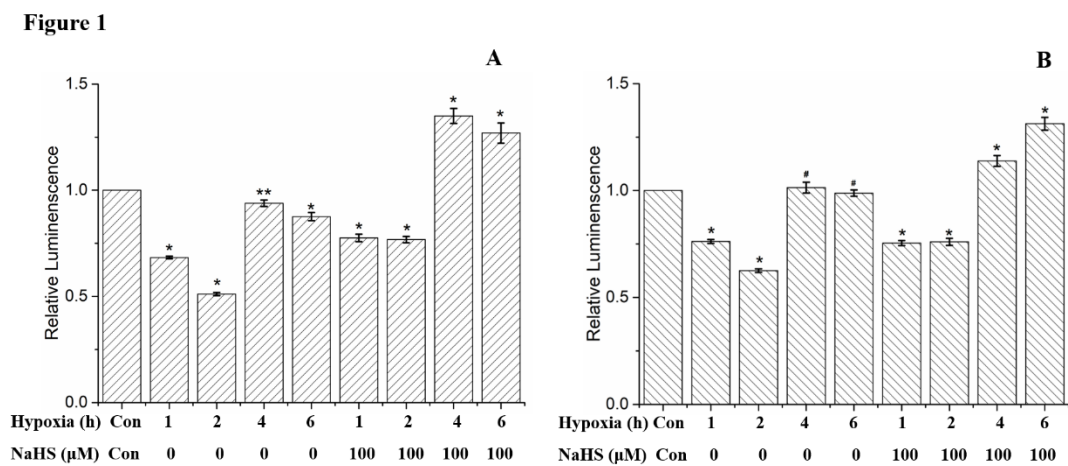



Figure 1

Figure 2

Figure 2

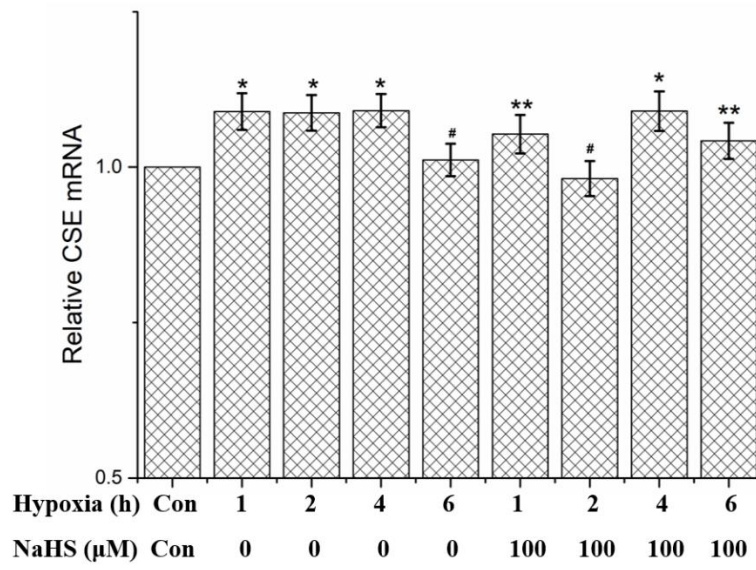
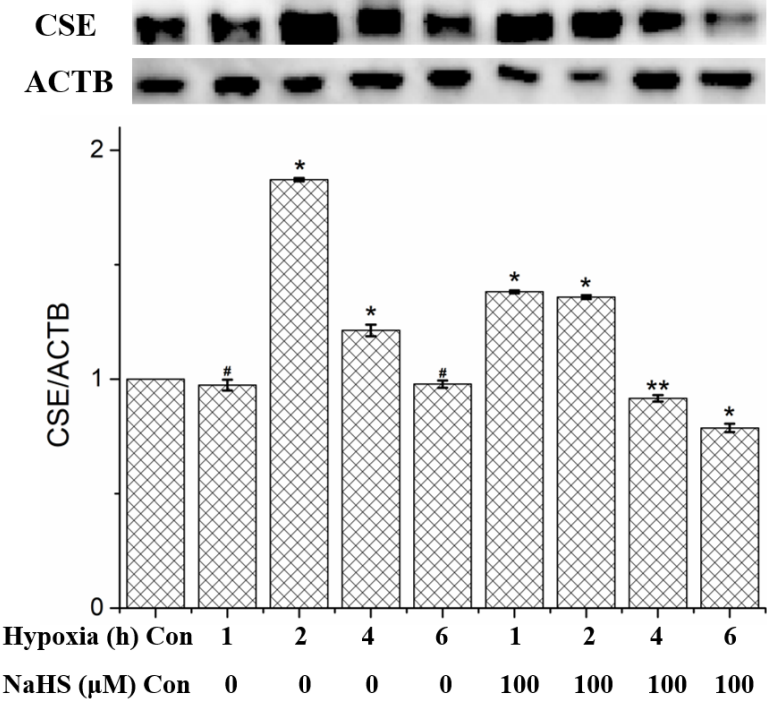



Figure 3

Figure 3

Additional files

Not applicable