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Cystathionine Gamma-Lyase Expression Is Regulated by Exogenous Hydrogen Peroxide in the Mammalian Cells

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Hydrogen sulfide (H_2S), as an endogenous signaling molecule in mammals, shows a variety of biological effects. Cystathionine gamma-lyase (CSE)/H_2S pathway has been implicated in scavenging reactive oxygen species (ROS) in the mammalian cells. Therefore, we first investigated the regulatory effects of exogenously applied hydrogen peroxide (H_2O_2) on CSE expression in the mammalian cells. African green monkey kidney fibroblast-like cells (COS-7 cells) or human embryonic kidney 293 cells (HEK 293 cells) were transfected with CSE promoter-luciferase reporter constructs and treated with H_2O_2 of 1, 5, and 10 μM for 0.5 and 1.5 h at 37°C. The transfected cells were assayed for *firefly* luciferase activities normalized by *Renilla* luciferase activity. Human lung adenocarcinoma cells (A549 cells) or human liver cancer cells (SMMC-7721 cells) were treated with H_2O_2 of 1, 5, and 10 μM for 0.5 and 1.5 h at 37°C, and were then harvested and analyzed by Western blotting and quantitative RT-PCR. Our results showed that the treatment of a medium concentration (5 μM) of H_2O_2 at a longer time (1.5 h) upregulated CSE expression in the mammalian cells at the levels of the promoter, message RNA, and protein. Collectively, exogenously applied H_2O_2 can not only markedly affect CSE mRNA and protein expression, but also can affect the CSE promoter activity in the mammalian cells. Our observations indicate that that exogenous H_2O_2 can upregulate the expression of the CSE gene in the mammalian cells, which will provide the possibility of the scavenging effect of the CSE gene indirectly on ROS in the mammalian cells. However, the regulatory mechanism involved in the effects of exogenously applied H_2O_2 on CSE expression in the mammalian cells need be further studied.

Key words: Hydrogen sulfide; Cystathionine gamma-lyase; Hydrogen peroxide; Oxidative stress

INTRODUCTION

Hydrogen sulfide (H_2S), as an endogenous signaling molecule, has been proven to be produced endogenously in the mammalian cells and plays important roles in physiological and pathophysiological conditions (5,9,23,27). Cystathionine gamma-lyase (CSE), one of three key enzymes in the transsulfuration pathway, is responsible for the production of endogenous H_2S (19,21,23,26,28–30). Some evidence suggests that H_2S exerts protective effects against various stimuli-triggered injuries in many organs including heart, liver, and kidney (7,20,22). One of the most important mechanisms responsible for H_2S protection is antioxidation, which exerts its effect not only by increasing reduced glutathione (GSH) in neurons (14), but also by directly scavenging superoxide anions, hydrogen peroxide (H_2O_2) (10).

A state of moderately increased levels of intracellular reactive oxygen species (ROS) is referred to as oxidative stress such as H_2O_2. H_2O_2 may be separately regulated, although it can cause pathological damage; both also function in normal cell regulation (4,18). The antioxidative effect of H_2S has been demonstrated in a variety of cell models (10,14,16). Administration of exogenous H_2S effectively protects

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myocytes and contractile activity by its direct scavenging of ROS (10). The H_2S administration restored the level of enzyme activities and accelerated the scavenging of H_2O_2 and superoxide anion generated by homocysteine in isolated mitochondria (2). H_2S protects neurons from oxidative stress by increasing the production of the antioxidant glutathione (14), and protects cells against H_2O_2-induced oxidative injury (16,25).

Since H_2S is a reducing agent that readily reacts with H_2O_2, it is possible that endogenous H_2S can scavenge ROS (14). However, it is not known whether or not exogenous H_2O_2 can regulate CSE expression in the mammalian cells. Therefore, we investigated the regulatory effects of exogenously applied H_2O_2 on CSE expression in the mammalian cells.

MATERIALS AND METHODS

Construction of a Luciferase Reporter Under the Control of CSE Promoter

A 1.7-kb DNA fragment upstream of the transcriptional start site (−1691 to +25) of the CSE gene was isolated by PCR using the genomic DNA of adult Kunming mice/SLAC (Shanghai SLAC Laboratory Animal Co. Ltd, Shanghai, China) as the template with the two primers: KM1716-forward (5′-CGGGG TACCTCCTGGATTCACATACTCCTTTG-3′) and KM1716-reverse (5′-CCGCTCGAGAGGAGTGCG AGGTGTTGCTTTGGCT-3′). The reaction conditions consisted of the followings: 3 min of initial preheating at 94°C, 30 cycles of 95°C for 30 s, 56°C for 45 s, and 72°C for 2 min 30 s, and a final elongation at 72°C for 10 min. The PCR product was digested with *Kpn*I (TAKARA Biology Co., Ltd, Dalian, China) and *Xho*I (TAKARA Biotechology Co.), subcloned into the promoterless pGL4.12 vector (Promega, Madison, WI, USA) that contains a firefly luciferase gene driven by the promoter activity of inserted sequences, and sequenced for confirmation, producing the pGL4.12-KM1716 vector. The inserted DNA fragment was confirmed by DNA sequencing (Biosune Biotechnology Co., Ltd. Shanghai, China).

Cell Culture and Treatments of Exogenously Applied H_2O_2

Human embryonic kidney 293 cells (HEK 293 cells) were cultured in a 5% CO_2/balance air incubator at 37°C in a DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and with an additive of 100 U/ml penicillin G, 100 μg/ml streptomycin, and 6.5 mM L-glutamine. African green monkey kidney fibroblast-like cells (COS-7 cells) and human liver cancer SMMC-7721 cells (SMMC-7721 cells) were cultured in a 5% CO_2/balance air incubator at 37°C in a RPMI-1640 medium supplemented with 10% heat-inactivated FBS, with an additive of 100 U/ml penicillin G, 100 μg/ml streptomycin, and 2 mM L-glutamine. Human lung adenocarcinoma A549 cells (A549 cells) were cultured in a 5% CO_2/balance air incubator at 37°C in a DMEM/F12 medium supplemented with 10% heat-inactivated FBS, with an additive of 100 U/ml penicillin G, 100 μg/mL streptomycin, and 4.5 mM L-glutamine.

According to the different cell lines, the transfected HEK 293 cells, the transfected COS-7 cells, A549 cells, and SMMC-7721 cells were seeded at a density of $0.5–1 \times 10^6$ cells in 35-mm dishes (Corning Glass, Corning, NY, USA) prior to treatment. H_2O_2 (3%, Sigma-Aldrich, Product No.: 323381) was first diluted to 100 μM, 500 μM, and 1 mM of H_2O_2 with double distilled water by the gradient dilution method. Taking 20 μl solution of H_2O_2 into 2 ml medium, the concentration of H_2O_2 in the cell medium was 1, 5, and 10 μM, respectively. Therefore, all tested cells were treated with H_2O_2 for 1, 5, and 10 μM for 0.5 and 1.5 h at 37°C. The control group received the same volume of saline as that of the H_2O_2. After treatment, the cells were immediately lysed and assayed. Luciferase assay, quantitative real-time PCR, and Western blotting were as described below.

Luciferase Assay

Because the DNA transfection efficiency would be dependent on the different cells, we decided to regard COS-7 cells and HEK 293 cells as the cell type transfected. The reporter gene assay was conducted in transiently transfected HEK 293 cells and COS-7 cells that were maintained in cell medium. When HEK 293 cells and COS-7 cells cultured in 35-mm dishes (Corning Glass) were 70–80% confluent, 0.032 μg (HEK 293 cells) or 0.0032 μg (COS-7 cells) of the pRL-CMV vector that contains a *Renilla* luciferase gene and 5 μg of the pGL4.12-KM1716 vector were combined, and then the DNA mixture was incubated with Xfect™ transfection reagent (Clontech Laboratories, Inc., CA, USA) at the ratio of 1.5 μl of Xfect™ polymer/5 μg of DNA. Transfection was performed according to the manufacturer's instructions. After 12 h, the transfected cells were subcultured in several 35-mm dishes at the proportion of 1:3. After exogenously applied H_2O_2 was used to treat the transfected COS-7 cells of 0.5×10^6 cells/35-mm dish or HEK 293 cells of 1×10^6 cells/35-mm dish, the transfected cells were assayed for measuring the *firefly* and *Renilla* luciferase activities after 48 h of DNA transfection, which was measured using the

Dual-Luciferase® Reporter Assay (Promega) according to the manufacturer's instructions with the SpectraMax® microplate reader (Molecular Devices Corporation).

Quantitative Real-Time PCR

To investigate CSE expression in A549 cells or SMMC-7721 cells, total RNA was isolated using the TransZol Up Reagent (TransGen Biotech, Beijing, China) according to the manufacturer's instructions after the treatment cells were rinsed with 1× dPBS buffer twice. Obtained RNA samples were quantified by absorbance at 260 nm (Biophoto, Eppendorf) and the integrity, purity, and amount of RNA were verified by visualization of rRNAs after electrophoresis on agarose gel. Total RNA concentration was measured by fluorescence using a Qubit™ RNA Assay Kit (Invitrogen, Shanghai, China) according to the manufacturer's instructions. First-strand cDNA was synthesized by incubation at 42°C for 30 min using anchored oligo$(dT)_{18}$ primer. The reaction mixture was in a total volume of 20 µl containing 2 µg of RNA, 1 µl of anchored oligo$(dT)_{18}$, 10 µl of 2× TS Reaction Mix and TransScript™ RT/RI Enzyme mix as well as RNase-free water (TransGen Biotech, Beijing, China). The reaction was terminated by incubation at 85°C for 5 min and the reaction mixture could be stored at −20°C and used for RT-qPCR.

RT-qPCR was performed in a final volume of 25 µl containing 11 µl cDNA diluted with double-distilled H_2O (1:20), 0.5 µl of 0.2 µM different primer, 0.5 µl Passive Reference Dye II, and 12.5 µl of 2× TransStart™ Green qPCR SuperMix (TransGen Biotech, Beijing, China). All reactions were run in triplicate with an Agilent Mx3000P QPCR Systems (Agilent Technologies, USA) and the data were treated by the MxPro QPCR software (Agilent Technologies) using a SYBR green fluorescence quantification system with the following process: an initial denaturation step was made at 95°C for 10 min, and 45 cycles for 30 s at 95°C, 30 s at 60°C, and 10 s at 72°C. The primer pair Q CSE Forward Primer/Q CSE Reversed Primer (Table 1) was designed to determine the relative expression of CSE. The primers specific to our PCR template were treated by Blast biological 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 PCR reactions, respectively. Dissociation curves were made for each set of oligonucleotides to check primer specificity and to confirm the presence of a unique PCR product. To estimate PCR efficiencies, standard curves were made based on five serial dilutions of a cDNA stock. PCR efficiencies of all primer sets were between 95% and 100%. After verifying that *TUBA* (α-tubulin gene) and CSE primers had similar amplifying efficiencies, the comparative Ct method $2^{-\Delta\Delta Ct}$ was used to for performing relative quantification analysis of mRNA levels (17). The relative amount of each mRNA was normalized to the housekeeping gene, *TUBA*. Each sample was run and analyzed in triplicate. The average of the relative amount of each mRNA in control group is defined as 1.0. The quantity of transcripts was estimated from a standard line derived from 20-fold serial dilutions of cDNA pooled from A549 cells or SMMC-7721 cells.

Western Blotting Analysis

For total protein extraction, 1×10^6 cells (A549 cells or SMMC-7721 cells) were incubated in 120 µl of PIPA lysis buffer (mild) (Biomiga, Inc., San Diego, CA, USA), supplemented with 1 mM PMSF proteinase, 0.25 U/µl benzonase, inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Both cells and PIPA lysis buffer were incubated on ice for 30 min, and the lysate was cleared by centrifugation at 12,000 × *g* at 4°C for 15 min. After centrifugation, glycerol, 2-mercaptoethanol, 10% SDS, 1 M Tris-HCl (pH 6.7), and bromophenol blue were added to the supernatant, and then denatured at 95–100°C for 10 min. The proteins were separated by electrophoresis on a 10% [for the detection of CSE, and α-tubulin (B-7)] sodium dodecylsulfate (SDS)-polyacrylamide gel (Sigma-Aldrich) and transferred onto the polyvinylidene difluoride (PVDF) membrane (0.45 µm, Immobilon-P, Millipore, Billerica, MA, USA). The membranes were blocked with a blocking solution containing 5%

TABLE 1
PRIMERS USED FOR QUANTITATIVE REAL-TIME PCR ASSAYS

Gene	Gene Bank Accession No.	Forward/Reverse Primer	Exon	Amplicon Size
CSE	NM_001902.5	5′-CCTGGGCTGCCCTCTCATCCA-3′	2	183 bp
		5′-TGCCGGAAGCTCAGCAAGGC-3	2	
TUBA	NM_006009.2	5′-GCGAAGCAGCAACCATGCGTGA-3′	2	115 bp
		5′-CCATCGGGCTGGATGCCGTG-3	2	

skim milk, 137 mM NaCl, 0.1% Tween 20, and 20 mM Tris-HCl (pH 7.6). After a wash, the membrane was incubated overnight at 4°C with anti-CSE (30.7, Santa Cruz, Santa Cruz, CA, USA) monoclonal antibodies (1:1,000 dilution) or anti-α-tubulin (B-7, Santa Cruz) monoclonal antibodies (1:15,000 dilution). After another wash, the membrane was incubated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (1:10,000) (Santa Cruz) (for the detection of CSE and α-tubulin). Positive bands for CSE or α-tubulin were identified around 43–47 or 52–58 kDa, respectively, by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, IL, USA). The resulting films (Kodak Company Limited, Xiamen, China) were scanned and quantified using density metric analytical software (the Bio-Rad Quantity One software, Bio-Rad Laboratories Co., Ltd., CA, USA).

Statistical Analysis

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

RESULTS

Exogenously Applied H_2O_2 Had Little Effect or Induced the Transcription of the CSE Gene Depending on Cell Type

To investigate the effect of exogenously applied H_2O_2 on the transcription of the CSE gene by the luciferase activity was analyzed. As shown in Figure 1A, H_2O_2 treatment slightly decreased the luciferase activity at a short time (0.5 h) in the transfected HEK 293 cells. A more significant effect was observed at a longer time (1.5 h). Both low (1 μM) and high (10 μM) concentration of H_2O_2 decreased the luciferase activity, while the medium concentration (5 μM) of H_2O_2 had less effect, suggesting that the medium concentration of H_2O_2 probably induced an upregulation to counteract the downregulation. As shown in Figure 1B, the upregulation effect caused by the medium concentration (5 μM) of H_2O_2 was more significant in the transfected COS-7 cells at a longer time (1.5 h).

Exogenously Applied H_2O_2 Increases Total CSE mRNA Level

The effect of oxidative stress on CSE expression at the transcription level was also analyzed by RT-qPCR. As shown in Figure 2, the endogenous CSE mRNA level was significantly increased by the treatment of medium concentration (5 μM) of H_2O_2 in both A549 cells (Fig. 2A) and SMMC-7721 cells (Fig. 2B) at a longer time (1.5 h). In contrast, either a high concentration (10 μM) or low concentration (1 μM) of H_2O_2 had less effect, suggesting the medium concentration of H_2O_2 probably induced an upregulation to counteract the downregulation. A high concentration of H_2O_2 probably damaged the upregulation due to its high toxicity, while a low concentration of H_2O_2 was not enough to start the activation.

Exogenously Applied H_2O_2 Induces CSE Protein Level

The effect of exogenously applied H_2O_2 on CSE expression at the protein level was analyzed using the Western Blotting. As shown in Figure 3, the similar as the effect of oxidative stress at the transcription level, the CSE protein level was markedly increased by the treatment of a medium concentration (5 μM) of H_2O_2 in both A549 cells (Fig. 3A) and SMMC-7721 cells (Fig. 3B) at a longer time (1.5 h). In contrast, either a high concentration (10 μM) or low concentration (1 μM) of H_2O_2 had less effect, suggesting that the upregulation of CSE expression could be activated only when the H_2O_2 accumulated to a threshold.

DISCUSSION

The relevant studies indicated that exogenously applied H_2S can protect mammalian cells from H_2O_2-induced oxidative injury (5,7,19,30). In the present study, we first investigated the effects of exogenously applied H_2O_2 on CSE expression in several mammalian cell lines, and demonstrated that exogenous H_2O_2 was involved in the regulation of the CSE gene at the promoter, mRNA, and protein levels.

In recent years, both COS-7 cells and HEK 293 cells (two mammalian kidney cell lines) have not only been successfully used in the study of the CSE/H_2S signal pathway (11), but also with high DNA transfection efficiencies in gene transfection experiment. Some typical mammalian cell lines such as A549 and SMMC-7721 cells are frequently used in the experiment study of the CSE/H_2S signal pathway by many researchers (1,3,6,12,30–32). Therefore, both A549 cells and SMMC-7721 cells are good models for testing the posttranscriptional regulation of the CSE gene by exogenous H_2S in the mammalian cells, and both COS-7 and HEK 293 cells in the transcriptional activity.

In particular, it is noteworthy that the treatment of a medium concentration (5 μM) of H_2O_2 at a longer time (1.5 h) upregulated CSE expression in the mammalian cells at levels of the promoter, mRNA, and protein. Previous studies have reported that exposure

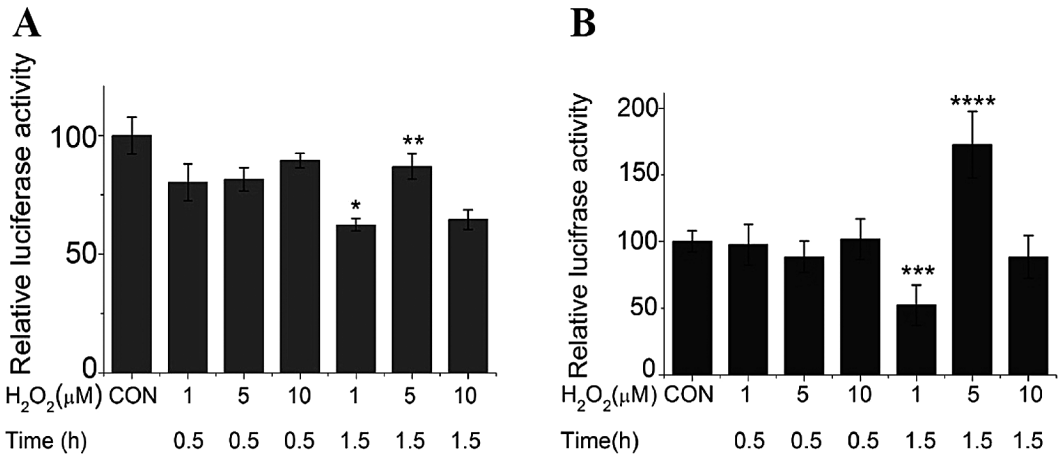

Figure 1. The effects of exogenous H_2O_2 on CSE gene expression at the promoter level. (A) Both low (1 μM) and high (10 μM) concentration of H_2O_2 decreased the luciferase activity in the transfected HEK 293 cells, while the medium concentration (5 μM) of H_2O_2 had less effect. (B) The upregulation effect caused by the medium concentration (5 μM) of H_2O_2 was more significant in the transfected COS-7 cells at a longer time (1.5 h). *$p < 0.01$ versus 5 μM + 1.5 h; **$p < 0.05$ versus 1 μM + 1.5 h; ***$p < 0.05$ versus 5 μM + 1.5 h; ****$p < 0.05$ versus 1 μM + 1.5 h.

of mammalian cells to H_2O_2 significantly increased ROS generation and H_2O_2 markedly suppressed intracellular GSH production in the mammalian cells (16). However, exogenous H_2S attenuates H_2O_2-induced ROS generation (16,25) and markedly reduced ROS accumulation in the mammalian cells (14,19,22). Although there is no consensus on the levels of H_2S in plasma (8,15,24) and the release of H_2S from tissue and cell production at the site of its function has not been measured (13), it can be speculated that the upregulation of CSE expression induced by exogenous H_2O_2 may be useful for increasing the production and concentration of endogenous H_2S, partly scavenging ROS such as H_2O_2 in the mammalian cells.

Collectively, exogenously applied H_2O_2 can not only markedly affect CSE mRNA and protein expression, but also can affect the CSE promoter activity in the mammalian cells. Our observations indicate that that exogenous H_2O_2 can upregulate the expression of the CSE gene in the mammalian cells, which will provide the possibility of the scavenging effect of the CSE gene indirectly on ROS in the mammalian cells. However, the regulatory mechanism involved in the effects of exogenously applied H_2O_2 on CSE expression in the mammalian cells need be further studied.

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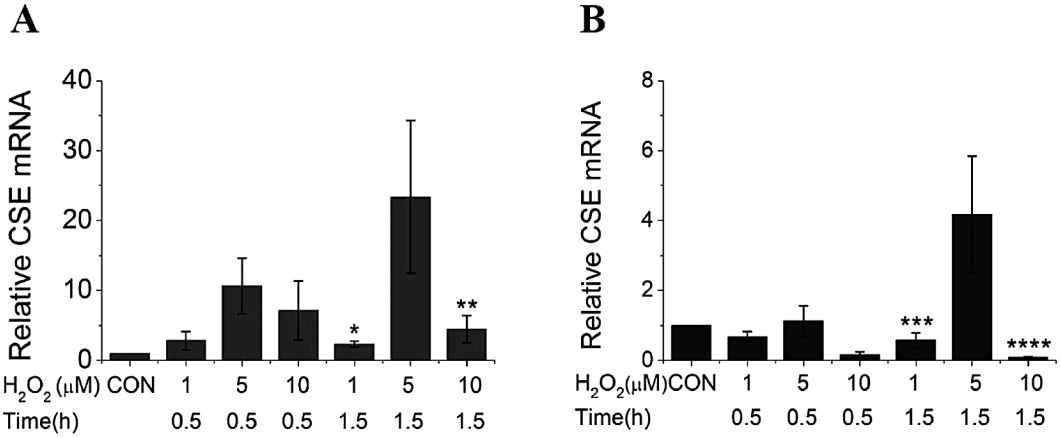

Figure 2. The effects of exogenous H_2O_2 on CSE gene expression at the mRNA level. The endogenous CSE mRNA level was significantly increased by the treatment of medium concentration (5 μM) of H_2O_2 in both A549 cells (A) and SMMC-7721 cells (B) at 1.5 h posttreatment. *$p < 0.01$ versus 5 μM + 1.5 h; **$p < 0.05$ versus 5 μM + 1.5 h; ***$p < 0.05$ versus 5 μM + 1.5 h; ****$p < 0.01$ versus 5 μM + 1.5 h.

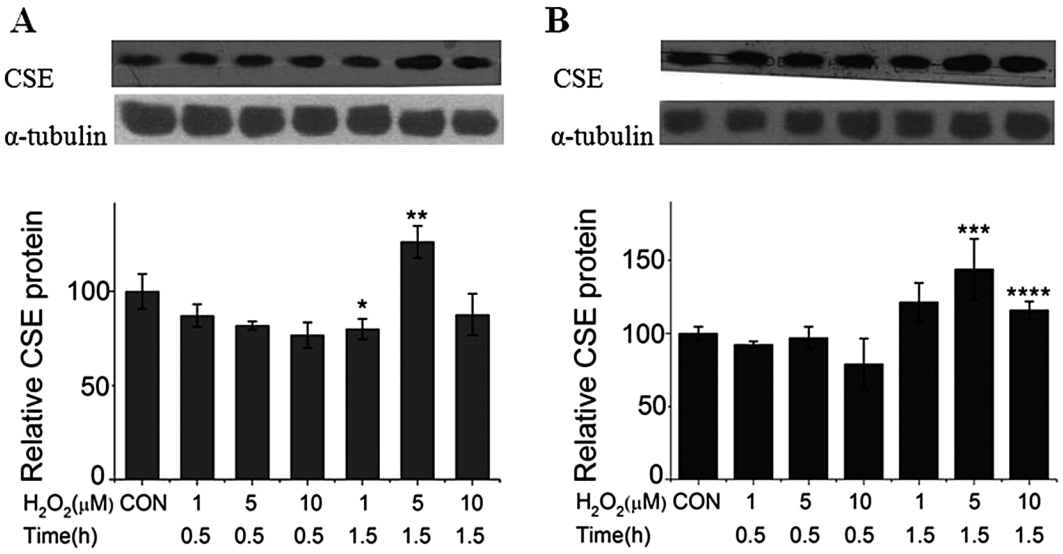

Figure 3. The effects of exogenous H_2O_2 on CSE gene expression at the protein level. Similar to the effect of H_2O_2 at the transcription level, the CSE protein level was also increased by the treatment of medium concentration (5 μM) of H_2O_2 in both A549 cells (A) and SMMC-7721 cells (B) at a longer time (1.5 h). *$p < 0.05$ versus 5 μM + 1.5 h; **$p < 0.05$ versus 1 μM + 1.5 h; ***$p < 0.05$ versus 1 μM + 1.5 h; ****$p < 0.05$ versus 5 μM + 1.5 h.

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