TNFα regulates the expression of the CSE gene in HUVEC

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Abstract. The hydrogen sulfide (H_2S)/cystathionine γ-lyase (CSE) signaling pathway is involved in several inflammatory conditions, where tumor necrosis factor-α (TNFα) is one of the inflammatory cytokines activated during sepsis. Therefore, the present study investigated the role of the NF-κB transcription factor binding site in the transcriptional regulation of the CSE gene in 293T cells following treatment with TNFα using luciferase assays, as well as using western blotting and reverse transcription-quantitative PCR to examine the effect of TNFα on CSE expression in HUVECs. After transfected 293T cells were incubated with various concentrations of TNFα for 1, 3, and 6 h, the wild-type promoter of the CSE gene increased significantly at 1 h compared to 0 h. By contrast, after the transfected 293T cells were incubated with various concentrations of TNFα for 1 h, the mutant-type promoter activity of the CSE gene decreased significantly compared to the wild-type. These results revealed that the DNA sequence GGGACATTCC on the CSE gene promoter was directly associated with the transcriptional regulation of the CSE gene in Human cells (293T cells) that's were treated with TNFα. This suggests that TNFα affects CSE gene expression, such that vascular endothelial cells respond to TNFα in the blood by regulating CSE expression. The regulatory mechanisms associated with the effects of TNFα on the transcriptional regulation of the CSE gene in HUVECs and the NF-κB pathway warrant further investigation.

Introduction

Cystathionine γ-lyase (CSE) is one of the three enzymes in the transsulfuration pathway that is responsible for producing endogenous hydrogen sulfide (H_2S) (1). NaHS (an H_2S donor) activates p38 and Akt, increasing the expression of angiogenic factors and the proliferation of HUVECs (2). H_2S is a gaseous endogenous mediator that serves a potential role in modulating gastric inflammatory responses (3). In particular, S-propargyl-cysteine is a H_2S donor that can enhance human umbilical vein endothelial cell (HUVEC) cell proliferation, adhesion, migration and tube formation (4). By contrast, exogenous thiosulfates, which act in a slow manner to modulate sulfide metabolites, have been documented to inhibit vascular endothelial growth factor (VEGF)-dependent endothelial cell proliferation in a manner that is associated with reductions in CSE protein levels (5). H_2S has been shown to inhibit the activation of NF-κB and the production of tumor necrosis factor-α (TNFα) in cultured uterine smooth muscle cells (6). Following CSE knockdown, treatment with L-aspartate-β-hydroxamate, an aspartate aminotransferase (AAT) inhibitor that blocks the generation of endogenous sulfur dioxide (SO_2)/AAT induced, was found to aggravate the activation of the NF-κB pathway in endothelial cells and its downstream inflammatory factors, including TNFα and interleukin (IL)-6 (7). H_2S exerts both pro-and antinociceptive effects through inflammation (8). In addition, a previous study observed an increase in inflammatory cytokine (IL-6 and TNFα) expression in the heart, and acute kidney injury (AKI) can downregulate CSE-mediated H_2S production, reduce glutathione levels and increase oxidative stress in the heart (9).

NF-κB is a heterodimer that is involved in a variety of signaling pathways (10). As such, NF-κB can regulate inflammatory responses by inducing the expression of a number of genes, such as IL-6 and intercellular adhesion molecule-1 (11), whilst NaHS suppresses intracellular adhesion molecule-1 expression in TNFα-treated HUVECs (12). TNFα is the multifunctional cytokine that is secreted primarily by macrophages, natural killer (NK) cells and lymphocytes (13). H_2S can prevent an endothelial monolayer activation triggered by TNFα. The mechanism of this protective effect is mainly mediated by downmodulation of ADAM17-dependent TNF-converting enzyme activity with consequent inhibition of soluble TNF-a shedding and its relevant MCP-1 release in the medium (14). CSE knockdown has been shown to protect primary hepatocytes from D-galactosamine/TNFα-induced cell death without affecting LPS-induced TNFα production by primary peritoneal macrophages (15). LPS induces significant increases in the plasma levels of multiple cytokines (TNFα, IL-1β, IL-6, IL-10, IL-12 and interferon γ), but TNFα, IL-10 and IL-12 levels tended to be lower in male WT mice (C57/BL6) compared with heterozygous cystathionine β-synthase (CBS) mice and CSE-knockout mice, which express lower H_2S-producing enzymes CBS, CSE and

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3-mercaptopyruvate sulfurtransferase (16). The findings indicate that CSE ameliorates the outcome of LPS-induced endotoxemia *in vivo* (16). By contrast, exogenous H_2S can attenuate angiotensin II-induced inflammation and cytotoxicity by inhibiting the endothelin 1/NF-κB signaling pathway in HUVECs (17). CSE-generated H_2S has also been shown to promote prostate cancer progression and metastasis through the IL-1β/NF-κB signaling pathway (18). In addition, H_2S can regulate lipopolysaccharide (LPS)-induced inflammation and apoptosis of the mammary alveolar epithelial cell lines, MAC-T by activating the PI3K/Akt/NF-κB signaling pathway (19).

The inflammatory cytokine TNFα serves a pivotal role in the disruption of macrovascular and microvascular circulation both *in vivo* and *in vitro* (20). Although the H_2S/CSE signaling pathway is involved in inflammation (19,21-23), mechanism underlying regulation of the CSE gene in HUVECs following treatment with TNFα remains poorly understood. Therefore, the aim of the present study was to investigate the role of the NF-κB transcription factor binding site on the transcription of the CSE gene in HUVECs that were treated with TNFα.

Materials and methods

Construction of the luciferase reporter under the control of human CSE promoter. HUVECs were obtained from the School of Pharmacy of Fudan University. The cultured cells were maintained in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific, 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. HUVECs were cultured to a confluence of 80-90% and digested with trypsin at 5,000 g/5 min with RT. The cells were collected into a 1.5 ml centrifuge tube. Subsequently, genomic DNA was extracted from the HUVECs using a blood genomic DNA extraction kit (Beijing Transgen Biotech Co., Ltd.). For identification, 1% agarose gel electrophoresis was used. The sequence of the human CSE gene promoter was searched on the GenBank database, based on which the upstream and downstream primers were designed. The target fragment DNA length was 710 bp (-696±16 nt). According to the GenBank database of the human CSE (accession no. NG_008041.1) gene promoter sequences and the Primer-BLAST online application (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), a 710-bp DNA transcription start site of human CSE gene was amplified by PCR using the HUVEC genomic DNA as the template (forward, 5'-CGGGGTACCCATTAGGGGGAGTTTCTC TCTGT-3' and reverse, 5'-CCGCTCGAGCTGCAGTCTCAC GATCACAGT-3'; Promega Corporation). The PrimeSTAR HS DNA Polymerase PCR kit (cat. no. R010A) was purchased from Takara Bio, Inc. The thermocycling conditions were as follows: Initial denaturation at 94˚C for 3 min; followed by 30 cycles at 95˚C for 30 sec, 60˚C for 45 sec and 72˚C for 90 sec; and a final step at 72˚C for 10 min. The PCR product was digested with the restriction enzymes *Kpn*I and *Xho*I (Takara Biotechnology Co., Ltd.) and cloned into the pGL4.12 vector (Promega Corporation) that containing a firefly luciferase gene driven by the inserted promoter (pGL4.12). The resultant construct was designated as pGL4.12-HuCSE710. The inserted DNA fragment was confirmed by Sanger sequencing by Sangon Biotech Co., Ltd. The reporter with the mutant CSE promoter was the same as that aforementioned, except that an alternative forward primer (5'-CGGGGTACCCATTAGGAT CTGTTTCTCTCTGT-3') was used during PCR amplification. Both the Wild-type promoter and the Mutant promoter were transformed into Trans5α chemically competent cells (A strain of *Escherichia coli* (*E. coli)*; TransGen Biotech Co., Ltd.), cultured overnight at 37˚C before a single bacterial colony was selected, followed by culture overnight in a shaking bed at 37˚C for amplification. Plasmids were extracted using the EasyPure Plasmid MiniPrep kit (cat. no. EM101-01; TransGen Biotech Co., Ltd.), which was analyzed by restriction enzyme digestion. The fragment size was identified and sequenced by Sanger sequencing by Sangon Biotech Co., Ltd. The sequencing results were compared with the published sequences on the GenBank database for analysis.

Cell culture and treatments. 293T cell lines (cat. no. GNHu17) were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cell culture reagents were purchased from Thermo Fisher Scientific, Inc. The 293T cells and HUVECs were cultured in a 5% CO_2/balance air incubator at 37˚C in DMEM supplemented with 10% heat-inactivated FBS and with the addition of 100 U/ml; penicillin G, 100 μg/ml streptomycin and 6.5 mM L-glutamine. According to the different cell lines, the transfected 293T cells and HUVECs were cultured at a density of 0.5-1x10^6 cells in 35-mm dishes (Corning, Inc.) prior to treatment. For treatment with TNFα (Recombinant Human 4-1BB Receptor, 5 μg; Beyotime Institute of Biotechnology), all tested cells were incubated with TNFα (10, 30 and 50 ng/ml) for 1, 3 and 6 h at 37˚C. The controls received the same volume of saline as the TNFα-treated cells. Following incubation and the removal of the cell medium, luciferase assay, reverse transcription-quantitative (RT-qPCR) and western blot analysis were performed.

Luciferase assay. For transfection, the 293T cells were grown to 70-80% confluency. Subsequently, 5 μg pGL4.12-HuCSE710 or pGL4.12-HuCSE710 m (the mutant promoter) together with 0.028 μg the pRL-CMV control vector were transfected into the cells per 35-mm dishes using Xfect™ transfection reagent (Takara Bio, Inc.) according to the manufacturer's protocols. After 12 h, the transfected cells were sub-cultured in several 35-mm dishes at a proportion of 1:3 for 24 h. Following treatment with TNFα for 1, 3, and 6 h after 36 h of transfection, both Firefly luciferase and *Renilla* luciferase activities were assayed using the TransDetect Double-Luciferase Reporter Assay kit (Beijing Transgen Biotech Co., Ltd.) according to the manufacturer's protocol using the Multimode Microplate Reader (Berthold Technologies GmbH & Co., K.G.). Firefly luciferase activity was normalized against *Renilla* luciferase activity.

Isolation of RNA and RT-qPCR. According to the manufacturer's protocol, total RNA of HUVECs was isolated using the TransZol Up reagent (Beijing Transgen Biotech Co., Ltd.). Reaction Mix and TransScript® RT/RI Enzyme mix (Transgen Biotech Co., Ltd.) was used for reverse transcription. The temperature protocol for reverse transcription was as follows: Incubation at 42˚C for 30 min and inactivation at 85˚C for

Table I. Primers used for reverse transcription-quantitative PCR.

Gene	GenBank Accession number	Primer sequence	Exon	Amplicon size
CSE	NM_001902.5	F:5'-GGCTCTACCTGCGTGCTTTA-3'	1	118 bp
		R:5'-CGCGAAAGAAGAAGAGAGGA-3'	1	
ACTB	NM_001101.3	F:5'-CTCTTCCAGCCTTCCTTCCT-3'	2	109 bp
		R:5'-TGTTGGCGTACAGGTCTTTG-3'	2	

CSE, cystathionine γ-lyase; ACTB, β-actin.

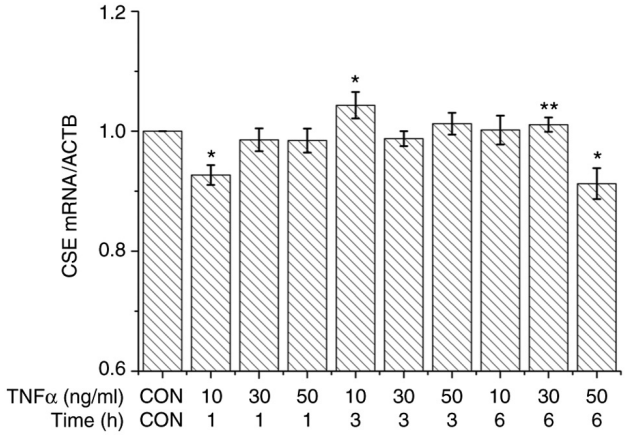

Figure 1. Effects of TNFα on the CSE gene transcription in HUVECs at the mRNA level. Following treatment with TNFα (10, 30 and 50 ng/ml) for 1, 3 and 6 h, mRNA expression levels of CSE in HUVECs were examined using reverse transcription-quantitative PCR. CSE mRNA levels in HUVECs decreased with 10 ng/ml TNFα for 1 h compared to all other except at 50 ng/ml for 6 h. Moreover, the CSE mRNA levels in HUVECs increased following treatment with TNFα at 10 ng/ml for 3 h. Compared with those in the control, CSE mRNA levels in HUVECs increased with TNFα at 10 ng/ml for 3 h, did not change significantly or declined in other cases (*P<0.05 and **P<0.01 vs. CON). CSE, cystathionine γ-lyase; HUVECs, human umbilical vein endothelial cells; TNFα, tumor necrosis factor α.

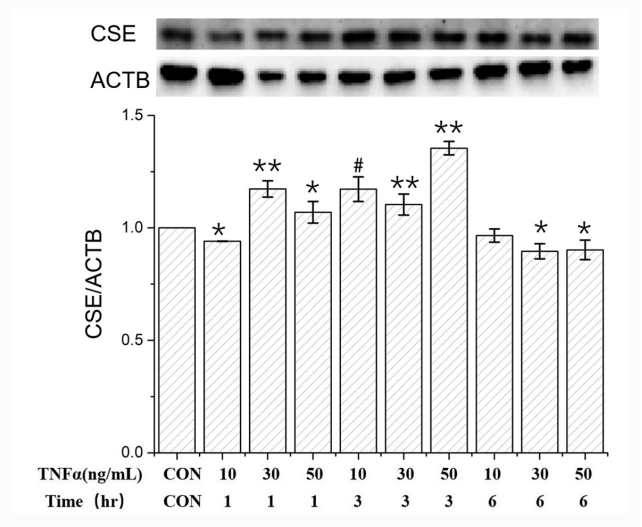

Figure 2. Effects of TNFα on the CSE expression in HUVECs at the protein level. Following treatment with TNFα (10 ng/ml) for 1 h, CSE protein expression significantly decreased compared with that in the control. Following treatment with TNFα (10, 30 and 50 ng/ml) for 1, 3 and 6 h, CSE protein expression in HUVECs increased at the concentration of 10 ng/ml with 3 h of treatment compared with 10 ng/ml after 1 h, whilst the CSE protein level in the HUVECs were increased at the concentration of 50 ng/ml for 3 h treatment compared with 50 ng/ml after 6 h. Compared to the control, CSE protein expression in the HUVECs markedly increased with TNFα at 30 ng/ml for 1 h and 50 ng/ml for 3 h but declined slightly in other cases (*P<0.05 and **P<0.01 vs. CON; $^{\#}$P<0.05, 10 ng/ml for 3 h vs. 10 ng/ml for 1 h). HUVECs, human umbilical vein endothelial cells; TNFα, tumor necrosis factor α; CSE, cystathionine γ-lyase.

5 sec. TransStart® Green qPCR SuperMix Real-Time PCR kit (Beijing Transgen Biotech Co., Ltd.) was used for qPCR. The following thermocycling conditions were used: Preincubation at 94˚C for 30 sec, followed by a 2-step amplification of 94˚C for 5 sec and 60˚C for 30 sec. An oligo-dT primer (Beijing Transgen Biotech Co., Ltd.) was used for mRNA reverse transcription. The sequences of the primers used for qPCR are summarized in Table I. To design the primer pairs, CSE Forward Primer/CSE Reversed Primer (Table I) was used to determine the relative expression of CSE. By verifying that both *ACTB* (β-actin gene) and *CSE* mRNA primers had similar amplifying efficiencies, the comparative $2^{-\Delta\Delta Cq}$ method was used for performing relative quantification analysis of the mRNA levels (24). Roche LightCycler® 96 System (Roche Molecular Diagnostics) was used for cDNA amplification and detection.

Western blot analysis. Western blot analysis was performed according to a previously described method (25). For total protein extraction, $0.5x10^6$ HUVECs were incubated in 120 μl mild RIPA lysis buffer (Beijing Transgen Biotech Co., Ltd.) supplemented with 1 mM PMSF proteinase, 0.25 U/μl Benzonase and inhibitor cocktail (Takara Bio, Inc.). The level of protein was determined using the bicinchoninic acid assay method. For 10% SDS-PAGE, the protein samples (30 μg) were mixed with loading buffer and boiled at 98˚C for 10 min. The separated proteins were transferred onto a 0.45 μm PVDF membrane (EMD Millipore). The membrane was incubated at 4˚C with anti-CSE (cat. no. D199513, Sangon Biotech Co., Ltd.) mouse monoclonal antibodies (1:1,000 dilution) or anti-ACTB (cat. no. D191047, Sangon Biotech Co., Ltd.) mouse monoclonal antibodies (1:2,000 dilution) for 12 h. After another wash, the membrane was incubated with HRP-conjugated goat anti-mouse antibodies (1:5,000 dilution; cat. no. D110087; Sangon Biotech Co., Ltd.) for 2 h, The results were scanned for the detection of CSE and ACTB with BeyoECL Plus (cat. no. P0018S; Beyotime Institute of Biotechnology) and quantified using the AlphaView System (ProteinSimple) sensitive chemiluminescent imaging system

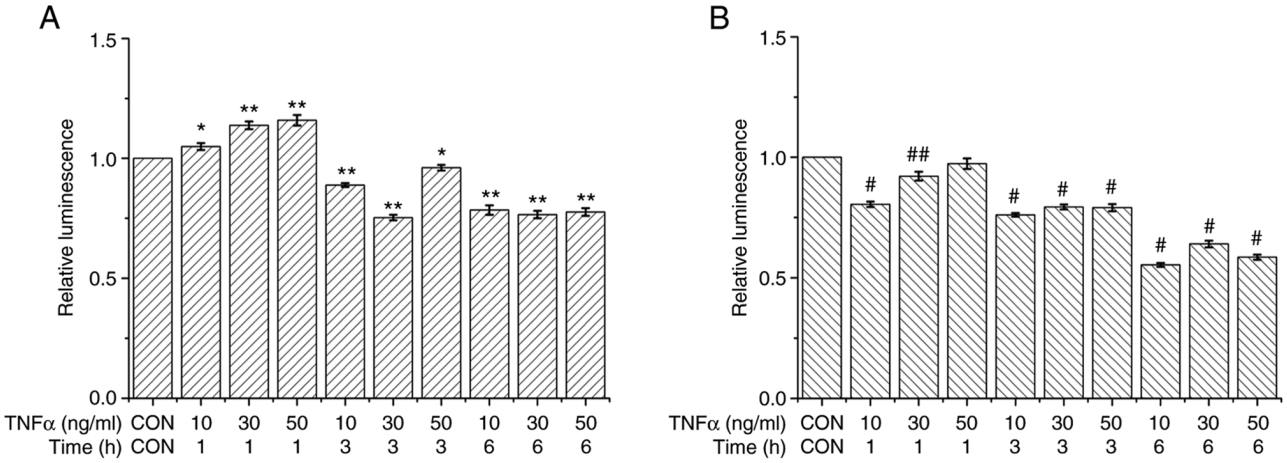

Figure 3. Effects of TNFα on the wild-type or mutant-type promoter luciferase activity of the CSE gene in transfected 293T cells treated with TNFα (10, 30 and 50 ng/ml) for 1, 3 and 6 h. (A) Wild-type promoter activity of the CSE gene. *P<0.05 vs. CON and **P<0.01 vs. CON (B) Mutant-type promoter activity of the CSE gene. $^{\#}$P<0.01 vs. CON and $^{\#\#}$P<0.01 vs. CON. CSE, cystathionine γ-lyase; HUVECs, human umbilical vein endothelial cells; TNFα, tumor necrosis factor α.

with a separate instrument software (FluorChem HD2; v3.4.0; ProteinSimple).

Statistical analysis. All data are expressed as the means ± SEM from ≥ four experiments. Shapiro-Wilk test is used to confirm the normality of the data distribution. Multiple group comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of TNFα treatment on the CSE mRNA level. To analyze the effects of TNFα on the transcription of the CSE gene, the expression level of CSE mRNA in HUVECs was examined. As shown in Fig. 1, following treatment with TNFα (10, 30 and 50 ng/ml) for 1, 3 and 6 h, the CSE mRNA levels in HUVECs decreased with 10 ng/ml TNFα for 1 h compared to all other groups except 50 ng/ml for 6 h. In addition, CSE mRNA levels in HUVECs increased following treatment with TNFα at 10 ng/ml for 3 h compared to all other groups. However, CSE mRNA levels of cells treated with higher concentrations of TNFα (50 ng/ml) were reduced at 3 h compared with that at 6 h, where it probably exerted toxic effects on the cells. At 3 h, a 'U' curve in CSE mRNA expression was observed in the cells treated with ascending concentrations of TNFα. At 6 h, an inverted 'U' curve was observed in the expression of CSE mRNA in the cells treated with ascending concentrations of TNFα. This result indicated that HUVECs treated with various concentrations of TNFα differed significantly among the different treatment times.

Effects of TNFα on the CSE protein level. The effect of TNFα on CSE protein expression in HUVECs was next investigated. As shown in Fig. 2, following treatment with TNFα (10 ng/ml) for 1 h, CSE protein expression decreased significantly compared with that in the control group. Following treatment with TNFα (10, 30 and 50 ng/ml) for 1, 3 and 6 h, the CSE protein level in the HUVECs increased, particularly at the concentration of 10 ng/ml for 3 h of treatment compared with that for 1 h. Compared with that in the control, CSE protein expression in the HUVECs increased with TNFα at 30 ng/ml for 1 h and 50 ng/ml for 3 h but declined slightly in other conditions compared to all other groups except 10 ng/ml for 3 h. These results suggested that the same concentration of TNFα exerted differential effects on the CSE protein level in a manner that was dependent on the treatment duration. Although TNFα-induced upregulation of CSE expression within 3 h at 50 ng/ml TNFα was evident compared to all other groups, TNFα can affect CSE expression in a concentration and duration-dependent manner.

Effect of TNFα on the wild-type or mutant promoter activity of the CSE gene. The effects of TNFα on the transactivation activities of the promoter of the CSE gene were subsequently analyzed by transient transfection experiments. By bioinformatics analysis, a potential NF-κB binding site was identified on the human CSE gene promoter with the DNA sequence of 5'-GGGACATTCC-3'. The reporter luciferase expression vector was constructed, which was either controlled either by the wild-type (Fig. 3A) or mutant (Fig. 3B) promoter region of the CSE gene, as shown. As shown in Fig. 3A, following treatment with TNFα (10, 30 and 50 ng/ml) for 1, 3 and 6 h, the wild-type promoter activity of the CSE gene in transfected 293T cells increased with the increment in the concentration of TNFα at 1 h compared to all other group.

As shown in Fig. 3B, the CSE gene mutant promoter activity decreased with the concentrations of TNFα at 1 h compared to TNFα (30 and 50 ng/ml) for 1, 3 and 6 h. These results revealed that TNFα regulated CSE gene transcription via the NF-κB binding site on the CSE gene promoter in HUVECs. Just as CSE mRNA expression was different following treatment with different concentrations of TNFα, the activity of CSE promoter was also similar at varying concentrations of TNFα. These results suggest that the level of TNFα at different treatment times affected the expression of CSE.

Discussion

The H_2S/CSE signaling pathway is involved in various inflammatory conditions, whereas TNFα is one of the inflammatory cytokines that is activated during sepsis (26-29). The concentration of H_2S is enhanced when abdominal sepsis or endotoxemia occurs, and administration of H_2S leads to exacerbation of these conditions, mainly because of its pro-inflammatory effect (30,31). It has also been reported that the concentration of H_2S rose in response to the presence of pancreatitis, which is ascribed to its pro-inflammatory effect (32) NaHS treatment also exerts anti-inflammatory effects through the inhibition of nitric oxide and TNFα production in MC3T3-E1 osteoblastic cells, suggesting an anti-inflammatory effect of H_2S (33). The anti-inflammatory effect of H_2S has been previously reported, particularly on TNF production, in addition to the molecular mechanisms involving NF-κB inhibition and the reduced expression of IL-6, TNF and IL-1β by H_2S (34). For example, H_2S production facilitates the pathogenesis of severe acute pancreatitis (35). Additionally, NaHS treatment has been shown to improve wound healing in ob/ob mice (hyperphagic, obese, hyperinsulinemic and hyperglycemic mice), which is associated with the decreased production of TNFα and IL-6 (36). The inflammatory role of H_2S remains controversial due to findings that it exerts both proinflammatory and anti-inflammatory effects (37).

TNF-α induces the gene expression of various inflammatory cytokines and chemokines, such as IL-6, IL-8 and MCP-1, either dependently or independently to activate transcriptional factors, such as NF-κB and activator protein 1 (20). Results from the present study demonstrated that transcriptional regulation of the CSE gene was possibly mediated on the NF-κB binding site on the promoter in HUVECs after treatment with TNFα. After the transfected 293T cells were incubated with TNFα (10 ng/ml) for 1, 3 and 6 h, the wild-type promoter activity of the CSE gene significantly increased at 1 h. However, the mutant-type promoter activity of the CSE gene considerably decreased at 1 h. When comparing the action of the wild-type promoter and the mutant-type, it was found that the CSE gene promoter activity significantly decreased with the mutation at 10, 30 and 50 ng/ml for 1 h. CSE expression was upregulated at a low concentration of TNFα over a short time period, whereas it was suppressed with the high concentration of TNFα and a longer treatment time. This suggest that TNFα exerted a concentration- and time-dependent effect on CSE expression. In HUVECs, the high concentration (50 ng/ml) of TNFα and a longer treatment duration (6 h) downregulated CSE expression, which may reduce endogenous H_2S production and induce the inhibition of vascular smooth muscle relaxation (38) resulting in reduced local blood flow (39). This may lead to the chronic insufficiency of blood supply to local tumor tissue, resulting in the induction of apoptosis (19) and in the prevention of tumor recurrence, this suggests a potential therapeutic target for tumor (40). These results demonstrated that the DNA sequence, GGGACATTCC, on the CSE gene promoter was directly associated with the transcriptional regulation of the CSE gene in mammalian cells treated with TNFα. In the present study, the effect of TNFα on the transcription regulation and expression of the CSE gene in HUVECs and 293T cells. However, further studies in animal models are required in future experiments.

In conclusion, results from the present study suggest that the NF-κB binding site on the CSE promoter is potentially important for TNFα-induced CSE expression. TNFα can potentially affect CSE gene expression, where vascular endothelial cells can respond to TNFα in the blood by regulating the CSE gene expression. Consequently, the regulatory mechanisms associated with the effects of TNFα on the transcriptional regulation of the CSE gene in HUVECs via the NF-κB pathway warrant further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MW contributed to this study independently; read and approved the final version of the manuscript; and confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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