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Inhibition of hydrogen sulfide production by gene silencing attenuates inflammatory activity of LPS-activated RAW264.7 cells

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Abstract Hydrogen sulfide is an inflammatory mediator and is produced by the activity of the enzyme cystathionine γ-lyase (CSE) in macrophages. Previously, pharmacological inhibition of CSE has been reported to have conflicting results, and this may be due to the lack of specificity of the pharmacological agents. Therefore, this study used a very specific approach of small interfering RNA (siRNA) to inhibit the production of the CSE in an in vitro setting. We found that the activation of macrophages by lipopolysaccharide (LPS) resulted in higher levels of CSE mRNA and protein as well as the increased production of proinflammatory cytokines and nitric oxide (NO). We successfully used siRNA to specifically reduce the levels of CSE mRNA and protein in activated macrophages. Furthermore, the levels of proinflammatory cytokines in LPS-activated macrophages were significantly lower in siRNA-transfected cells compared to those in untransfected controls. However, the production levels of NO by the transfected cells were higher, suggesting that CSE activity has an inhibitory effect on NO production. These findings suggest that the CSE enzyme has a crucial role in the activation of macrophages, and its activity has an inhibitory effect on NO production by these cells.

Keywords Hydrogen sulfide · siRNA · Cystathionine gamma lyase · Inflammation · Cytokines · Nitric oxide

Introduction

There is growing evidence that hydrogen sulfide (H_2S) performs a wide range of physiological and pathological functions. It is now increasingly being accepted as the third endogenous gaseous mediator alongside carbon monoxide and nitric oxide (NO). Endogenous hydrogen sulfide is produced from L-cysteine mainly by the activity of two enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). In general, endogenous hydrogen sulfide production in the periphery depends on CSE (Moore et al. 2003), while CBS is primarily expressed in the central nervous system (Enokido et al. 2005; Zhao et al. 2007). Recent reports showed that the production of hydrogen sulfide in macrophages depends solely on CSE and that following this production, hydrogen sulfide acts as an autocrine and signals further activation of macrophages (Miller et al. 2012; Oh et al. 2006; Zhu et al. 2010). This suggests that hydrogen sulfide may have an important role in the signaling of inflammation.

As an advanced protective process, inflammation is initiated in response to tissue injury and infection. Macrophages have a crucial role in initiating and propagating inflammation. Proinflammatory mediators are released by activated macrophages to signal the recruitment and activation of leukocytes. However, uncontrolled and excessive release of inflammatory mediators and the release of free radicals by leukocytes are implicated in pathological inflammation (Bellingan 2000). An in vitro study on human monocytes demonstrated that elevated levels of hydrogen sulfide induced cytokine secretion (Zhi et al. 2007). The mechanism by which hydrogen sulfide induces these effects needs further investigation. Early research points to many potential pathways in which hydrogen sulfide induces its effect including NO, inducible nitric oxide synthase (iNOS), substance P, and nuclear factor kappa B (Ang et al. 2011; Benetti et al. 2013; Geng et al. 2007; Oh et al. 2006; Zhang et al. 2007). Possible interaction between hydrogen sulfide and NO is controversial. There are some reports on the inhibitory effects of NO on hydrogen sulfide production, whereas an in vivo study demonstrated stimulatory effects

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of NO on hydrogen sulfide production (Anuar et al. 2006; Oh et al. 2006). As hydrogen sulfide has been shown to modulate the immune system's activity, there is a therapeutic potential for intervention in endogenous hydrogen sulfide production during inflammatory diseases.

Hydrogen sulfide is an inflammatory mediator, and its levels increase following inflammation. Inhibition of hydrogen sulfide production using DL-propargylglycine (PAG), a CSE inhibitor, resulted in the reduced levels of hydrogen sulfide produced by pancreatic acinar cells and significant reduction of the severity of cerulein-induced pancreatitis and associated lung injury (Bhatia et al. 2005b; Tamizhselvi et al. 2008). Currently, the most used CSE inhibitor, PAG, is plagued by specificity issues. Despite its inhibitory effect on hydrogen sulfide production, undesired effects of PAG are well documented. A metabolite of PAG is nephrotoxic and injures proximal tubular cells in the kidney (Konno et al. 2000). PAG also has been shown to alter amino acid metabolism as well as having other physiological effects such as polyuria, proteinuria, and glycosuria (Kawaji et al. 1992; Konno et al. 2000). A novel method of specifically inhibiting the action of CSE would be greatly beneficial to our understanding of the specific role of CSE in normal and pathological physiology; it may also be a potential therapeutic approach in pathologies involving inflammation such as pancreatitis, sepsis, and burn injury.

Small interfering RNA (siRNA) mediates the cleavage of the targeted mRNA and attenuates the production of specific target protein. Therefore, siRNA has promise as a novel therapeutic strategy and, in addition, may be used as a tool for functional genomics to elucidate genes controlling disease pathways (Aouadi et al. 2009; Peer et al. 2008). Genetical deletion of mCD14 gene by siRNA resulted in the lower levels of proinflammatory mediators and NO production in lipopolysaccharide (LPS)-activated RAW264.7 cells (Lei et al. 2011).

In this study, we determine the use of CSE targeting sequence-dependent siRNA as an effective approach to silence the CSE gene, and in consequence of CSE gene silencing, we investigate the levels of proinflammatory cytokines and NO production in LPS-activated RAW264.7 cells.

Materials and methods

Cell line and treatments

Murine macrophage cell line, RAW264.7 cells, was maintained at 37 °C in Dulbecco's modified Eagle's medium (Gibco BRL, USA) containing 10 % heat-inactivated fetal bovine serum (Gibco BRL, USA) supplemented with penicillin (100 U/ml; Gibco BRL, USA) and streptomycin (100 μg/ml; Gibco BRL, USA). Based on our preliminary findings, the levels of cytokine production and NO_x were measured and found to be at their optimum with LPS concentration of 100 ng/ml when measured 24 h after LPS administration. Therefore, this treatment paradigm was used. LPS (Sigma, cat. no. L-2630) was dissolved in saline to reach final growth media concentrations of 100 ng/ml.

siRNA transfection

siRNA transfection was performed with different siRNA concentrations, cell densities, and incubation times to determine the efficient dose of siRNA to silence the CSE gene. Reverse transfection was performed with the Lipofectamine RNAiMAX (Invitrogen) with a 24 h incubation in serum-free media (Opti-MEM, Invitrogen) as per the manufacturer's instruction. Cells (7×10^5/well) were seeded in six-well plate. At 24 h posttransfection, media were changed with standard media containing serum and antibiotic, and wells designated to the activated group were treated with LPS (100 ng/ml) for 24 h. The growth media were stored at −80 °C for analysis. Transfection efficiency was confirmed using the Block-iT Alexa Fluor Red Fluorescent Oligo (Invitrogen) as per the manufacturer's instruction.

Cell viability assay

The 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) test was performed to assess the cell viability. Briefly, cells (2×10^4) were seeded in 96-well plates. At 24 h post-siRNA transfection, cells were treated with LPS for 24 h. Following LPS treatment, MTT solution (20 μl per 100 μl medium) was added to the wells and incubated at 37 °C for 4 h. Isopropanol acid (100 μl of 0.04 N HCL in isopropanol) was added to wells and then mixed thoroughly and incubated for 8 h. The plates were read on a machine using a test wavelength of 570 nm.

RNA extraction and quantitative real-time polymerase chain reaction

At 48 h posttransfection, TRIzol Reagent (Invitrogen) was used for total RNA extraction from RAW264.7 cells as per manufacturer's instructions; 5 μg RNA was reversely transcribed using the M-MLV First Strand Kit (Invitrogen) and stored at −20 °C. Real-time quantitative PCR was performed using the Eco Real-Time PCR System (Illumine). A comparative CT method was used to compare the CSE mRNA levels of RAW264.7 cells transfected with siRNAs and negative control.

All PCR reactions were normalized to the Ct value of beta-actin. Assays were triplicated, and results are shown as mean ± standard deviation. The sense and antisense primers were used for CSE, iNOS, and beta-actin (Table 1).

Table 1 PCR primer sequences, optimal amplification cycles, optimal annealing temperature, and product size

Gene	Primer sequence	Optimal condition	Size (bp)
CSE	Sense: 5-GCAATGGAATTCTCGTGCCG-3_ Antisense: 5-ATGCAAAGGCCAAACTGTGC-3′	25 cycles Annealing 60 °C	115
iNOS	Sense: 5-GCTCCCTATCTTGAAGCCCC-3 Antisense: 5-ACTGACACTTCGCACAAAGC-3′	28 cycles Annealing 62 °C	199
Beta-actin	Sense: 5-CTGTCGAGTCGCGTCCACCC-3 Antisense: 5-ACATGCCGGAGCCGTTGTCG-3′	20 cycles Annealing 60 °C	128

CSE protein expression determined by Western blot

Cells were harvested and lysed in lysis buffer consisting of 50 nM Tris–HCl, pH 8 (1 M); 150 mM NaCl (5 M); 10 % glycerol; 1 % Igapal; and 1 % protease inhibitor (Thermo Scientific). The cell lysates were vortexed vigorously for 15 s and were kept in 4 °C. The process was repeated three times every 15 min. The protein concentration was determined using the DC protein assay (Bio-Rad). Aliquots of protein were then separated on 10 % SDS polyacrylamide gels and subsequently transferred onto nitrocellulose membranes electrophoretically. The membranes were blocked for 1 h in 5 % nonfat milk in 0.05 % Tween 20 in PBS and were then incubated with the primary antibody raised against CSE (Abnova) at a dilution of 1:1,000 overnight at 4 °C. At the following day, the membranes were incubated with a secondary horseradish peroxidase-conjugated antibody at 1:10,000 dilutions for 1 h at room temperature. The enhanced chemiluminescence detection kit (PerkinElmer, USA) was used to visualize immunoreactive proteins. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Santa Cruz Biotechnology) was used as the housekeeping protein.

Enzyme-linked immunosorbent assay analysis

Enzyme-linked immunosorbent assay analysis (ELISA) was performed to determine the protein levels of the main proinflammatory cytokines (TNF-α, IL-6, IL-1β, and monocyte chemotactic protein (MCP)-1) secreted into the growth media. Supernatants were used for measuring the level of cytokines. The levels of TNF-α, IL-6, IL-1β, and MCP-1 were measured using the specific DuoSet ELISA Kit (R&D Systems, Minneapolis, MN) as per the manufacturer's instruction.

NO assay

NO levels were determined by measuring the total nitrite and nitrate content (nitric oxide metabolites) in growth media using the Griess assay (Ang et al. 2009). Briefly, the samples were incubated with 10 U/ml nitrate reductase (β-NADPH; Sigma), supplemented with 60 mM NADPH (Sigma) and 1 mM FAD (Sigma) for 30 min at 37 °C in the dark to convert exciting nitrate to nitrite. Griess reagent (1 % sulfanilamide, 0.1 % N-NED, 2.5 % H_3PO_4; Sigma) was added in equal volumes and incubated for 15 min in the dark. The absorbance was then measured at 540 nm, and the concentration was calculated using sodium nitrate standards.

Statistics

One-way ANOVA and Tukey's multiple comparison tests were used for statistical analysis. The experiments were performed in triplicate, and the results are expressed as mean ± SD. The p values of <0.05 was regarded as statistically significant.

Results

In preliminary experiments, RAW264.7 cells released the highest levels of cytokines when incubated with 100 ng/ml LPS for 24 h (data not shown). Therefore, at 24 h posttransfection of siRNA, cells were treated with 100 ng/ml LPS for 24 h. Different concentrations of siRNA were screened to evaluate CSE gene expression, and based on quantitative polymerase chain reaction (qPCR) results, the inhibitory effects of 50 nM of siRNA were the strongest (data not shown). Therefore, this dose of siRNA was selected in our experiments. The results of MTT determined that 50 nM of siRNA had no cytotoxic effects on the RAW264.7 cells. Cell viability for siRNA-transfected cells was 84 % of control, showing that CSE targeting with siRNA did not affect the cell viability.

Inhibition of CSE mRNA expression by siRNA

qPCR was performed to quantify CSE transcription in LPS-induced RAW264.7 cells transfected with siRNA and untransfected controls. There was a significant effect of LPS and siRNA treatment on CSE expression in RAW264.7 macrophages (F (2, 6)=27.76, $p<0.001$) (Fig. 1a). LPS treatment 24 h prior to qPCR resulted in a significantly higher level of CSE expression with levels 4.8 (1.2 SD) fold higher than those of the control ($p<0.01$) (Fig. 1a). siRNA administration reduced the effect of LPS on CSE expression to 1.2 (0.1 SD)-fold higher than that of the control ($p<0.01$) (Fig. 1a).

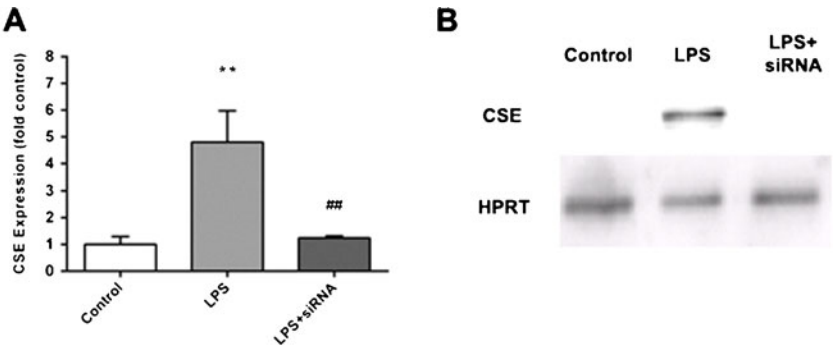

Fig. 1 The effects of LPS and siRNA on CSE mRNA (**a**) and protein (**b**) expression in RAW264.7 macrophages. CSE mRNA expression is expressed as a fold control. The control group (*white*) is untreated, the LPS group (*light gray*) has received 100 ng/l LPS 24 h prior to qPCR, and the LPS+siRNA group (*dark gray*) has received 50 μM siRNA 24 h prior to LPS treatment which was administered 24 h prior to qPCR (**a**). For all groups, $N=3$. Error is SD. $^{**}p<0.01$, compared to control; $^{\#\#}p<0.01$, compared to LPS using planned comparisons. Western blot analysis (**b**) showing protein expression in untreated RAW264.7 cells (**a**), LPS-treated cells (**b**), and siRNA-transfected cells treated with LPS (**c**) ($N=3$)

Inhibition of CSE protein expression by siRNA

Western blot analysis confirmed a single-protein band of approximately 43 kDa corresponding to the CSE protein. RAW264.7 cells treated with LPS expressed a higher level of CSE. Our results indicated that siRNA-targeted CSE had inhibitory effects on CSE protein expression. HPRT was used as a loading control to compare CSE expression (Fig. 1b).

The effect of CSE silencing on cytokine secretion by LPS-induced RAW264.7 cells

At 24 h posttransfection with siRNA targeting CSE, the cells were treated with LPS (100 ng/ml) for 24 h. The growth media were collected for cytokines analysis.

TNF-α, IL-1β, IL-6, and MCP-1

Analysis of the ELISA results demonstrated that the levels of the TNF-α, IL-1β, IL-6, and MCP-1 released to growth media by LPS-induced (100 ng/ml) RAW264.7 cells were significantly higher than untreated cells. Analysis of ELISA results demonstrated that the levels of TNF-α, IL-1β, IL-6, and MCP-1 in the LPS-induced (100 ng/ml) RAW264.7 cells pretreated with siRNA reduced significantly compared to untreated cells.

There was a significant effect of LPS and siRNA treatment on the levels of TNF-α secretion by RAW264.7 macrophages (F (2, 33)=72.08, $p<0.0001$) (Fig. 2a). LPS treatment 24 h prior to ELISA analysis resulted in a significantly higher level of the TNF-α secretion with measured concentration of 11.22 ng/ml (3.33 SD) compared to the control ($p<0.0001$) (Fig. 2a). siRNA administration significantly reduced LPS-induced TNF-α secretion to 7.78 ng/ml (2.3 SD) ($p<0.01$) (Fig. 2a). The effect of LPS and siRNA on the levels of IL-1β secretion by RAW264.7 cells was significant (F (2, 24)=54.81, $p<0.0001$ and $p<0.01$, respectively). The levels of IL-1β secretion by the LPS-treated cells were significantly higher than those by the control group with the measured concentration of 0.33 ng/ml (0.1 SD) ($p<0.0001$) (Fig. 2b). Administration of siRNA significantly prevented the effect of LPS on the levels of IL-1β secretion by RAW264.7 cells with the measured concentration of 0.11 ng/ml (0.045 SD) ($p<0.0001$) (Fig. 2b).

Our results showed that the treatment of RAW264.7 cells with LPS and siRNA had a significant effect on the levels of IL-6 secretion (F (2, 15)=191.7) (Fig. 2c). Treatment of cells with LPS for 24 h demonstrated higher levels of IL-6 secretion compare to that of the control with the measured concentration of 75.55 ng/ml (11.53 SD) ($p<0.0001$), while siRNA administration resulted in lower level of IL-6 secretion with the measured concentration of 13.70 ng/ml (2.8 SD) ($p<0.0001$) compare to the cells treated with LPS (Fig. 2c). LPS and siRNA treatment had a significant effect on the levels of MCP-1 secretion (F (2, 24)=75.23, $p<0.0001$) (Fig. 2d). Following incubation cells with LPS for 24 h, the levels of MCP-1 secretion were significantly higher with the measured concentration of 127.7 ng/ml (21 SD) compare to those of the control group ($p<0.0001$) (Fig. 2d). Incubation of RAW264.7 cells with LPS for 24 h, pretreated with siRNA, resulted in significantly lower levels of MCP-1 secretion with the measured concentration of 85.7 ng/ml (32.5 SD) ($p<0.0001$) compared to the LPS-treated group (Fig. 2d).

Effects of CSE silencing on NO production from LPS-treated RAW264.7 cells

We assessed NO production by measuring the total nitrite and nitrate content in growth media. At 24 h posttransfection with siRNA targeting CSE, the cells were then treated with LPS (100 ng/ml). At 48 h posttransfection, growth media were collected for NO production analysis. The results indicated that the LPS and siRNA had a significant effect on NO

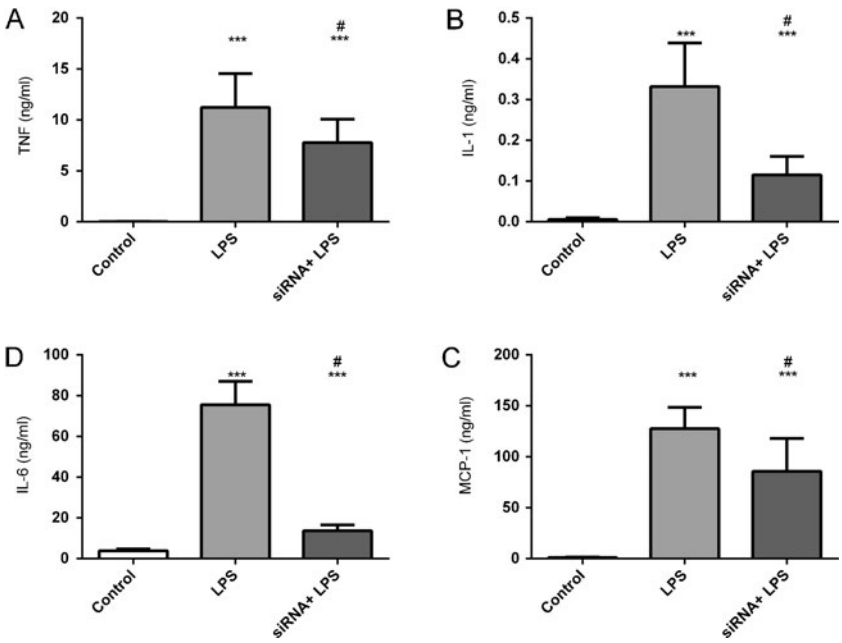

Fig. 2 siRNA against CSE significantly reduced LPS-induced cytokine production. The control group (*white*) is untreated, the LPS group (*light gray*) was treated with 100 ng/l LPS 24 h prior to ELISA assay, and 50 μM siRNA was transfected to LPS+siRNA group (*dark gray*) 24 h prior to LPS treatment which was administered 24 h prior to ELISA assay. CSE targeting siRNA effectively reduced the levels of LPS-induced TNF-α (**a**), IL-1β (**b**), IL-6 (**c**), and MCP-1 (**d**) production. For all groups, $N=3$. Error is SD. ***$p<0.0001$, compared to control; $^{\#\#}p<0.01$, compared to LPS using planned comparisons

production by RAW264.7 cells (F (2, 33)=752.2, $p<0.0001$) (Fig. 3). RAW cells treated with LPS released a higher level of the NO to the growth media with the measured concentration of 0.721 μM (0.056 SD) compared to 0.164 μM (0.007 SD) in the control (Fig. 3a). Administration of siRNA to the cells resulted in higher levels of NO production with the measured concentration of 0.81 μM (0.51 SD) ($p<0.0001$) compared to the LPS group (Fig. 3a).

Effects of CSE silencing on iNOS expression by LPS-treated RAW264.7 cells

qPCR was performed to quantify iNOS transcription in LPS-induced RAW264.7 cells transfected with siRNA and untransfected controls. There was a significant effect of LPS and siRNA treatment on iNOS expression in RAW264.7 macrophages (F (2, 6)=274.8, $p<0.00001$) (Fig. 3b). LPS treatment 24 h prior to qPCR resulted in a significantly higher level of iNOS expression with levels 11.4 (1.06 SD)-fold higher than those of the control ($p<0.00001$) (Fig. 3b). siRNA administration increased the effect of LPS on iNOS expression with levels 23.2 (1.41 SD)-fold higher than those of the control ($p<0.00001$) (Fig. 3b).

Discussion

Hydrogen sulfide is a biological mediator which plays an important role in normal physiology and disease state. Endotoxemia studies have shown that LPS administration

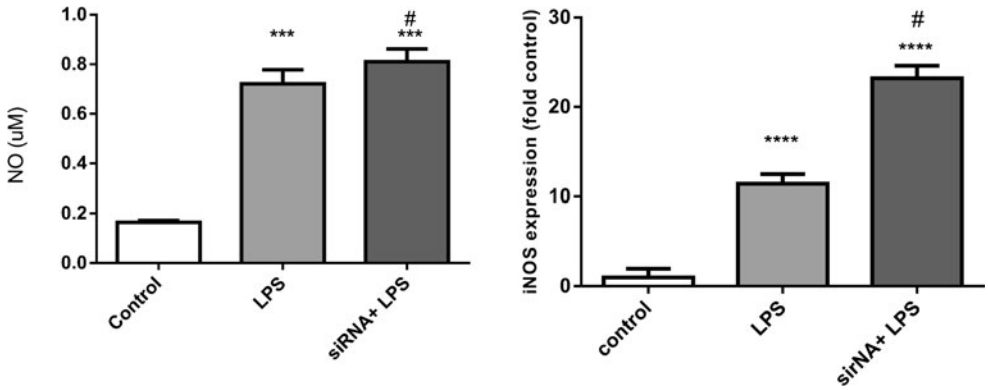

Fig. 3 CSE targeting siRNA effectively increased LPS-induced NO_x production (left) and iNOS expression (right). The control group (*white*) is untreated, the LPS group (*light gray*) was treated with 100 ng/l LPS 24 h prior to NO assay and 50 μM siRNA was transfected to LPS+siRNA group (*dark gray*) 24 h prior to LPS treatment which was administered 24 h prior to NO assay and qPCR. There was a significant increase in the level of NO_x production and iNOS expression by the LPS-treated RAW264.7 cells which were pretreated with siRNA. $^{\#\#\#\#}p<0.0001$ compared to LPS treated group; ****$p<0.0001$ compared to control group. For all groups $N=3$. Error is SD. Mean ± SD

results in heightened levels of hydrogen sulfide in the plasma as well as an upregulated CSE mRNA expression in the kidney (Li et al. 2005a). Inhibition of hydrogen sulfide production by synthetic drugs such as PAG ameliorates inflammatory disease, while administration of hydrogen sulfide donors exacerbated the severity of inflammatory disorders (Collin et al. 2005; Li et al. 2005b; Tamizhselvi et al. 2007).

Macrophages have been generally used as one of the cellular models to screen for potential anti-inflammatory treatment methods. They play an important role in the initiation and resolution of inflammation through presenting antigen and phagocytosis. They also play a key role in the expression of proinflammatory cytokines via proinflammatory signal transduction cascades, and because of this, these cells have been implicated as having a crucial role in inflammatory diseases (Fujiwara and Kobayashi 2005; Grip et al. 2003). In accordance to a previous report (Zhu et al. 2010), this study demonstrated that RAW264.7 cells express CSE mRNA, and activation of these cells with LPS resulted in increased expression levels of CSE mRNA and protein. In mammalian cells, hydrogen sulfide is produced by CSE and/or CBS. RAW264.7 cells, however, only express CSE (Zhu et al. 2010). Therefore, in these cells, hydrogen sulfide is only produced via CSE. Activated macrophages secrete higher levels of cytokines such as TNF-α, IL-1β, and IL-6 (Avni et al. 2010; Liu et al. 2003), which have been known to play important roles in proinflammatory responses. In agreement with previous reports, our results confirm increased levels of released proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and MCP-1 by RAW264.7 cells following stimulation with LPS (100 ng/ml).

It is well documented that overexpression of key proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 regulates the cascades of cytokines, chemokines, and adhesion molecule expression in many inflammatory disease (Coussens and Werb 2002; Dinarello 1991; Scheller et al. 2006). The beneficial effect of CSE inhibition has been reported in a number of animal models of inflammatory diseases (Bhatia et al. 2005a; Li et al. 2005a; Zhang et al. 2006). An in vivo study determined that the inhibition of hydrogen sulfide production ameliorated the severity of acute pancreatitis and associated lung injury (Bhatia et al. 2005b). Inhibition of hydrogen sulfide production by PAG, a CSE inhibitor, reduced the levels of MCP-1, macrophage inflammatory protein (MIP)-2, and MIP-1α (Tamizhselvi et al. 2008; Zhang et al. 2007). These reports point an important proinflammatory role for hydrogen sulfide, suggesting that hydrogen sulfide could promote inflammation through upregulation of chemokines.

Based on the specificity and potency, siRNA is an attractive method to silence the CSE gene. To explore the role of hydrogen sulfide in LPS-activated RAW264.7 macrophages, we used the Silencer siRNA (Invitrogen) to knockdown the CSE gene which is responsible for producing hydrogen sulfide. In the last few years, siRNA has been considered intensively with the hope of developing therapeutics in inflammatory diseases. siRNA has been shown to reduce profound inflammation specifically when it was used against mCD 14 in an in vitro (Lei et al. 2011) and MCP-1 receptors (CCR2) in an in vivo study (Leuschner et al. 2011).

In this study, we demonstrated that the inhibition of hydrogen sulfide production by macrophages using CSE-targeted siRNA could attenuate the inflammatory response by reducing the proinflammatory cytokines' production levels. Our results show that silencing CSE expression in RAW cells resulted in reduced production levels of TNF-α, IL-1β, IL-6, and MCP-1 in LPS-induced RAW264.7 cells. In this regard, the inhibitory effects of hydrogen sulfide on release of proinflammatory cytokines from RAW264.7 cells with siRNA could have an anti-inflammatory potential to treat many inflammatory disorders.

On the other hand, silencing CSE with siRNA resulted in increased levels of NO production and iNOS mRNA expression in LPS-induced RAW264.7 cells, suggesting inhibitory effect of hydrogen sulfide on NO production levels. Interaction between hydrogen sulfide and NO has been reported in several studies. Inhibitory effect of hydrogen sulfide on NO production in LPS-induced RAW264.7 cells was reported in an in vitro study (Oh et al. 2006; Zhu et al. 2010). In accordance with these reports, our findings showed that hydrogen sulfide has an inhibitory effect on NO production. Silencing the CSE mRNA which is the only enzyme expressed in RAW264.7 cells resulted in a significant increase in the levels of iNOS expression and NO production in these cells. In another study, Anuar et al. (2006) have shown that NO has an inhibitory effect on hydrogen sulfide production. They showed that administration of NO releasing nitroflurbiprofen to a rat pretreated with LPS resulted in decreased CSE expression and hydrogen sulfide production. Reducing the activity of NF-κB is a suggested mechanism for this effect of NO (Anuar et al. 2006). Inhibition of hydrogen sulfide production (Bhatia et al. 2005b) and increased iNOS expression and iNOS-derived NO (Hickey et al. 1997; Kobayashi et al. 2001) have been reported to protect lung injury, suggesting an interaction between NO and hydrogen sulfide and also a regulatory role for NO in inflammatory diseases. Absence of iNOS accelerates the inflammatory response in the lung which might relates to increased levels of MCP-1 production by macrophages, suggesting a regulatory role for iNOS in the production of chemokines by macrophages (Desai et al. 2003; Speyer et al. 2003). In agreement with this report, our results demonstrated that CSE silencing by siRNA enhanced the expression of iNOS and NO production and reduced the levels of cytokines and chemokines. These data suggest a regulatory role for iNOS in cytokine production. To our knowledge there has only been one study published the effect of CSE-targeted

siRNA on the levels of NO production and iNOS expression. In this study, siRNA-targeted CSE reduced hydrogen sulfide production, and in consequence, NO production levels and iNOS expression in LPS-induced RAW264.7 cells increased (Zhu et al. 2010). In accordance with this report, our findings also suggest that silenced CSE expression resulted in increased levels of NO production and iNOS expression in LPS-induced RAW264.7 cells. These findings suggest that hydrogen sulfide plays an inhibitory effect on NO production.

The present study also demonstrated that in subsequent CSE silencing, iNOS expression and NO production increased, and the levels of proinflammatory cytokines reduced in LPS-induced macrophages, suggesting that hydrogen sulfide has inhibitory effects on NO production. Therefore, inhibition of hydrogen sulfide production could ameliorate the inflammation.

In conclusion, this study shows that activation of macrophages by LPS increases CSE expression and proinflammatory cytokines levels. CSE-targeted siRNA successfully inhibited mRNA expression and lowered CSE protein levels. This resulted in a subsequent decrease in the production of proinflammatory cytokines and increased iNOS expression and NO production by macrophages. Our findings suggest that the inhibition of hydrogen sulfide biosynthesis by macrophages could be a therapeutic strategy to regulate uncontrolled activation of these cells in inflammatory disorders. We propose that the inhibition of hydrogen sulfide production could be used as a therapeutic approach in the inflammatory diseases. Further testing of the therapeutic potential of siRNA targeting CSE could be subject for future study.

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Conflict of interest The authors have no conflicts of interest to report.

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