Cell. Mol. Life Sci. (2010) 67:1119–1132
DOI 10.1007/s00018-009-0250-9

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Glucocorticoids suppress cystathionine gamma-lyase expression and H_2S production in lipopolysaccharide-treated macrophages

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Received: 14 September 2009 / Revised: 30 November 2009 / Accepted: 15 December 2009 / Published online: 10 January 2010

Abstract Hydrogen sulfide (H_2S) plays an important role in inflammation. We showed that macrophages expressed the H_2S-forming enzyme cystathionine gamma-lyase (CSE) and produced H_2S. Lipopolysaccharide (LPS) stimulated the CSE expression and H_2S production rate. L-cysteine reduced LPS-induced nitric oxide (NO) production. CSE inhibitor blocked the inhibitory effect of L-cysteine. CSE knockdown increased, whereas CSE overexpression decreased LPS-induced NO production. Dexamethasone suppressed LPS-induced CSE expression and the H_2S production rate as well as NO production. L-arginine increased, whereas N^G-nitro-L-arginine methyl ester (L-NAME) decreased LPS-induced CSE expression and H_2S production. Dexamethasone plus L-NAME significantly decreased LPS-induced CSE expression and H_2S production compared to L-NAME. Our results suggest that macrophages are one of the H_2S producing sources. H_2S might exert anti-inflammatory effects by inhibiting NO production. Dexamethasone may directly inhibit CSE expression and H_2S production, besides the NO-dependent way. Inhibition of H_2S and NO production may be a mechanism by which glucocorticoids coordinate the balance between pro- and anti-inflammatory mediators during inflammation.

Keywords Glucocorticoids · Hydrogen sulfide · Cystathionine γ-lyase · Lipopolysaccharide · Macrophages

Abbreviations

H_2S	Hydrogen sulfide
NO	Nitric oxide
CO	Carbon monoxide
CSE	Cystathionine gamma-lyase
CBS	Cystathionine β-synthetase
fMLP	Formyl-methionyl-leucyl-phenylalanine
TNF-α	Tumor necrosis factor-α
GCs	Glucocorticoids
GR	Glucocorticoid receptor
MIF	Migration inhibitory factor
LPS	Lipopolysaccharide
IL-1	Interleukin-1
IL-6	Interleukin-6
MTT	3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide
PAG	DL-propargylglycine
L-NAME	N^G-nitro-L-arginine methyl ester
iNOS	Inducible nitric oxide synthase
CD-FBS	Charcoal-stripped FBS
PLP	Pyridoxal-5′-phosphate
siRNA	Interfering RNA
RAW-EGFP-mCSE	CSE-overexpression RAW264.7 cell lines

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RAW-mCSE siRNA	CSE-knockdown RAW264.7 cell lines
TBST	Tris-buffered saline/Tween 20

Introduction

Hydrogen sulfide (H_2S) has recently been suggested to be "the third endogenous gaseous signaling transmitter" in addition to nitric oxide (NO) and carbon monoxide (CO) in mammalian tissues [1, 2]. H2S is naturally synthesized in the body from L-cysteine mainly by the activity of two enzymes, cystathionine γ-lyase (CSE, EC 4.4.1.1) and cystathionine β-synthetase (CBS, EC 4.2.1.22) [3]. In animal models, physiological levels of plasma H_2S concentration have been reported to vary from 30 to 300 μM [4–6], and in vivo basal concentrations of H_2S are markedly augmented under certain pathological conditions, such as endotoxemia and septic shock [6, 7].

H_2S has been indicated to play critical roles in inflammatory processes. It seems that H_2S exhibits either pro- or anti-inflammatory effects. It has been shown that exogenous H_2S provokes an inflammatory reaction in lung in normal mice [6] and induces the generation of pro-inflammatory cytokine in cultured human monocytes [8]. Inhibition of H_2S biosynthesis displays distinct anti-inflammatory activity as evidenced by the attenuation of the organ injury in cases including septic shock, endotoxemia and pancreatitis [6, 9, 10]. On the other hand, H_2S has also been reported to play an anti-inflammatory role during inflammation. H_2S can scavenge peroxynitrite [11], inhibit leukocyte adherence and infiltration [12, 13], suppress edema formation [13], inhibit formyl-methionyl-leucyl-phenylalanine (fMLP)-induced chemotaxis and degranulation of polymorphonuclear leukocytes [14], induce neutrophil apoptosis [15] and suppress LPS-induced tumor necrosis factor-α (TNF-α) production in microglial cells [16].

Glucocorticoids (GCs) are a class of steroid hormones with pleiotropic effects through interaction with glucocorticoid receptor (GR) in a broad range of cell types. It is well known that GCs have anti-inflammatory effects. They inhibit the expression and production of numerous inflammatory mediators, such as pro-inflammatory cytokines, prostaglandins and reactive oxygen and nitrogen species in various cell types [17]. GCs inhibit leukocyte migration to sites of inflammation and promote lymphocyte apoptosis [18, 19]. However, the role of GCs in the regulation of inflammation is not fully understood. GCs have also been shown to exert pro-inflammatory effects in some cases [20, 21]. It was reported that GCs enhanced migration of leukocytes from the bloodstream to wound sites [22], and low physiological doses of cortisol stimulated the pro-inflammatory cytokine migration inhibitory factor (MIF) production in macrophages [23]. Some studies also showed that GCs could inhibit apoptosis of human neutrophils, thereby contributing to inflammatory processes [24–26].

Macrophages are a heterogeneous population of mononuclear phagocytes found ubiquitously in the body. These cells play a crucial role in innate and adaptive immunity in response to microorganisms and are major contributors to the inflammatory response through generation of numerous pro-inflammatory mediators [27, 28]. Recently, it has been shown that CSE mRNA is induced by lipopolysaccharide (LPS) in cells in the macrophage cell line RAW264.7 [29]. However, the capacity of H_2S production in primary macrophages, the possible functions of endogenous H_2S and the consequent changes of H_2S production in response to inflammatory stimuli remain to be clarified. Moreover, macrophages are the target cells of GCs. GCs have been shown to inhibit the release of pro-inflammatory cytokines including TNF-α, interleukin-1 (IL-1) and interleukin-6 (IL-6) [17], but promote phagocytosis of apoptotic cells by macrophages [30]. In addition, GCs have also been shown to induce changed expression of many pro- and anti-inflammatory genes in human monocytes [31, 32]. Recently, Li et al. [33] reported that dexamethasone decreased the expression of CSE in isolated rat neutrophils. It is of interest to explore the regulation of H_2S production by GCs in macrophages.

In this study, we firstly elucidated the capacity of H_2S biosynthesis in macrophages by looking at the expression of H_2S synthetic enzymes in response to LPS and consequent H_2S production. Then, we explored the possible local functions of endogenous H_2S by studying the effects of endogenous H_2S on NO production. Finally, we investigated the effects of dexamethasone, a potent synthetic glucocorticoid, on the expression of H_2S synthetic enzymes as well as H_2S biosynthesis in LPS-treated macrophages.

Materials and methods

Materials

Purified LPS (*Escherichia coli*, O111:B4), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), sodium orthovanadate, pyridoxal-5′-phosphate, DL-propargylglycine (PAG),dexamethasone, RU38,486, L-cysteine, L-arginine and N^G-nitro-L arginine methyl ester (L-NAME) were obtained from Sigma-Aldrich (St. Louis, MO). TRIzol reagent and superscript reverse transcriptase were purchased from Invitrogen (Grand Island, NY). Antibody to CSE was purchased from Abnova (Taipei, Taiwan). Antibodies to CBS, inducible nitric oxide synthase (iNOS),

secondary horseradish peroxidase-conjugated antibody and the enhanced chemiluminescence Western blotting detection system were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

Peritoneal macrophages were obtained from male C57BL/6 mice, which were purchased from Shanghai SLAC Laboratory Animal Co. (Shanghai, China) and were 8–10 weeks of age when used in the study. The mice were housed in a temperature- and humidity-controlled environment and received standard mouse chow and water ad libitum in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). Also, all experimental procedures were approved by the Ethics Committee of Experimental Animals of the Second Military Medical University, China. Resident peritoneal exudate cells were washed from the peritoneal cavity with 2 ml ice-cold DMEM medium (Life Technology, Grand Island, NY) as described previously [34]. After centrifugation at $110 \times g$ for 10 min, the exudate cells were resuspended in DMEM medium and then plated in six-well tissue culture plates (Corning Glass, Corning, NY) at 1×10^6 cells per well in a final volume of 2 ml. Cells were incubated at 37°C for 2 h to adhere to plastic, and nonadherent cells were rinsed off by vigorously washing with DMEM medium containing 10% FBS. Viability of the adherent cells was >95% as assessed by trypan blue exclusion. Purity was determined by FACScan (Becton–Dickinson, San Jose, CA) with specific monoclonal antibody F4/80 (PharMingen, San Diego, CA), and about 95% of the adherent macrophages were found to express F4/80 marker.

Murine macrophage cell line RAW264.7 was originally obtained from American Type Culture Collection (Rockville, MD) and kindly provided by Shanghai Institute for Biological Sciences. Cells were cultured in DMEM medium containing 10% FBS at 37°C in 5% CO_2, 95% air; RAW264.7 cells were also seeded on six-well plates at a density of 1×10^6 cells/well. Both primary and RAW264 macrophages were cultured in DMEM medium containing 10% charcoal-stripped FBS (CD-FBS). Peritoneal macrophages and RAW264.7 cells were then exposed to LPS (10–1,000 ng/ml), dexamethasone (1–1,000 nmol/l), L-cysteine (1 mmol/l), PAG (1 mmol/l), L-arginine (0.1–1 mmol/l), L-NAME (0.25–1 mmol/l) or RU38,486 (1 μmol/l) for the indicated time period. Dexamethasone was first dissolved in absolute ethanol and diluted by the culture media to achieve a final ethanol concentration of less than 0.01%. Control media contained the same final solvent concentrations (0.01%). Each treatment was performed in triplicate for each preparation of cells.

Total RNA extraction, RT-PCR and quantitative real-time RT-PCR

Total RNA of cells was isolated by using TRIzol reagent according to the manufacturer's instructions; 2 μg RNA was reverse transcribed using superscript reverse transcriptase (Invitrogen) and stored at −20°C. The following sense and antisense primers were used: CSE (accession number NM_145953): 5′-GAGCCTGAGCAATGGAAT-3′ and 5′-GATGGGTAATCGTAATGGTG-3′. CBS (accession number NM_178224): 5′-GAACCAGACGGAGCAAACAG-3′ and 5′-CTTGAACACGCAGACGCCAC-3′.

PCR reaction solution consisted of 2.0 μl diluted RT-PCR product, 0.2 μM of each paired primer, 2.5 mM Mg^{2+}, 250 μM deoxynucleotide triphosphates, 2 U Taq DNA polymerase (Promega, Madison, WI) and 1 × PCR buffer. The PCR reaction was set at 94°C (45 s), 58°C (45 s) and 72°C (1 min) in a total of 40 cycles with a final extension step at 72°C for 10 min. Ten microliters of the reaction mixture were subsequently electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide using a 100-bp DNA ladder (Life Technology) to estimate the band sizes. As a negative control for all of the reactions, distilled water was used in place of cDNA. The identity of the PCR products was confirmed by sequencing. Sequence data were analyzed using Blast nucleic acid database searches from the National Centre for Biotechnology Information (NCBI).

Quantitative real-time PCR analysis was carried out in duplicates using Rotor Gene 3000 (Corbett Research, Sydney, Australia). The real-time PCR solution consisted of 40 ng diluted cDNA product, 0.1–0.3 μM of each paired primer, 2.5 mM Mg^{2+}, 100 μM deoxynucleotide triphosphates, 2 U Taq DNA polymerase and 1 × PCR buffer. SYBR green (BMA, Rockland, ME) was used as detection dye. Quantitative real-time PCR conditions were optimized according to preliminary experiments to achieve linear relationships between the initial RNA concentration and PCR product. The annealing temperature was set at 58°C, and amplification cycles were set at 40 cycles. The temperature range to detect the melting temperature of the PCR product was set from 60–95°C. Amplification of the housekeeping genes β-actin and GAPDH were measured for each sample as an internal control for sample loading and normalization. The melting curve was examined at the end of the amplification to ensure the specificity of PCR products. To determine the relative quantitation of gene expression for both target and housekeeping genes, the comparative Ct (threshold cycle) method with arithmetic formulae ($2^{-\Delta\Delta Ct}$) was used [35]. Because very similar data were obtained by using either β-actin or GAPDH as an

internal control, GAPDH was used for calculation of ΔCt in the presentation of results.

Western blot analysis

Cells were scraped off the plate in the presence of lysis buffer consisting of 60 mmol/l Tris-HCl, 2% sodium dodecyl sulfate (SDS), 10% sucrose, 2 mmol/l phenylmethylsulfonyl fluoride (Merck, Darmstadt, Germany), 1 mmol/l sodium orthovanadate and 10 μg/ml aprotinin (Bayer, Leverkusen, Germany). The cell lysates were quickly sonicated and centrifuged at 12,000×*g* for 5 min at 4°C. The supernatant was collected, and the protein concentration was assayed using a modified Bradford assay. The samples were diluted in sample buffer (250 mmol/l Tris-HCl (pH 6.8), containing 4% SDS, 10% glycerol, 2% *β*-mercaptoethanol and 0.002% bromophenol blue, and boiled for a further 5 min. Aliquots of proteins were separated by SDS-PAGE (10%) and subsequently transferred to nitrocellulose membranes by electroblotting. The membrane was blocked in 5% skim milk powder in 0.1% Tris-buffered saline/Tween 20 (TBST) at room temperature for 2 h, and then was incubated with antibody raised against iNOS or CBS at a dilution of 1:1,000, and CSE at a dilution of 1:2,000 overnight at 4°C. After three washes with TBST, the membrane was incubated with a secondary horseradish peroxidase-conjugated antibody for 1 h at room temperature. Immunoreactive proteins were visualized using the enhanced chemiluminescence Western blotting detection system. The light-emitting bands were detected with X-ray film. The resulting band intensities were quantified using an image scanning densitometer (Furi Technology, Shanghai, China). To control sampling errors, the ratio of band intensities for iNOS or CSE to *β*-actin was obtained to quantify the relative protein expression level.

Real-time H_2S production measurement

To define the real-time kinetics of H_2S production by macrophages, we used a miniaturized H_2S micro-respiration sensor (Model H_2S-MRCh, Unisense, Aarhus, Denmark) coupled to a Unisense PA2000 amplifier. The 100-μm diameter H_2S microsensor is based on the original design of the amperometric microsensor used to measure H_2S with high spatial resolution as described before [36, 37]. Since the H_2S microsensor was sensitive to pH, temperature and electrical interferences [37, 38], the real-time H_2S production measurements were performed in a temperature-controlled micro-respiration chamber (Unisense) containing 1 ml of stirred (160 rpm) culture media (pH 7.2) at 37°C inside a well-grounded Faraday cage. Macrophages were collected and washed in fetal bovine serum-free culture media with 20 mM Hepes substituted for sodium bicarbonate to prevent CO_2 bubble formation in the closed respirometer chamber. The final cell pellet was resuspended at a concentration of 1×10^7 cells/ml, and 0.1 ml cell suspension was injected into the respirometer chamber. In addition, to avoid spontaneous H_2S oxidation [39], nitrogen was used to deoxygenate the culture media in the respiratory chamber before the addition of macrophages. After the sensor signals stabilized, L-cysteine (1 mmol/l, the substrate of CSE) and pyridoxal-5′-phosphate (PLP, 1 mmol/l, a cofactor of CSE) were added to stimulate H_2S production. H_2S production rates were determined after the addition of substrate and cofactor at the initial steepest slopes of each trace [37]. Cell viability was assessed by trypan blue exclusion and remained at more than 90% throughout the experiments. The H_2S sensor was calibrated after each experiment with freshly prepared anoxic sodium sulfide stock solution (0–100 μmol/l) according to the manufacturer's manual, using the same buffer and conditions as the experiment.

NO production assay

NO production in supernatants was assessed by measuring nitrite/nitrate, the stable degradation product of NO as described previously [40]. In brief, Griess reagent was prepared by mixing equal volumes of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphtylethylenediamide just before use; 100 μl of Griess reagent was mixed with equal amounts of cell supernatants. After incubation at 37°C for 10 min, the OD value was measured using a Bio-Rad (Hercules, CA) microplate reader at 550 nm. Concentration of nitrite was assessed by reference to the sodium nitrite standard curve.

Establishment of stable CSE-overexpression and CSE-knockdown cell lines

The mouse CSE full-length cDNA was synthesized and cloned (Jinsite Biotechnology Corp., Nanjing, China) into pEGFP-N3 vector (BD Biosciences Clontech, Palo Alto, CA), generating a C-terminal EGFP-tagged fusion protein. The mouse CSE small interfering RNA (siRNA) plasmid was constructed using the pRNAT-U6.1/Neo vector (Jinsite Biotechnology) for knockdown of the CSE gene in vitro. The target sequence of mouse CSE-specific siRNA used in the present study was 5′-TACATGAATGGCCACAG CGAT-3′. Control-siRNA plasmid was supplied by Jinsite Biotechnology, and it expressed a hairpin siRNA with no homology to any known mouse or rat mRNA sequences in the NCBI RefSeq database.

RAW264.7 cells were separately transfected with pEGFP-N3, pEGFP-mCSE, pRNAT-U6.1/Neo-mCSE and pRNAT-U6.1/Neo-Control vector using SofastTM cationic

polymer transfection reagent (Sunma Biotech, Xiamen, China) according to the manufacturer's manual. Forty-eight hours after transfection, stable transfected cells were selected using 800 μg/ml G418 in growth medium for 2 weeks. Surviving clones were then incubated in medium containing 400 μg/ml G418. The stable CSE-overexpression and CSE-knockdown RAW264.7 cell lines were named "RAW-EGFP-mCSE" ("RAW-EGFP" as control) and "RAW-mCSE siRNA" ("RAW-control siRNA" as control), respectively.

Determination of CSE promoter activity

PGL_3-luciferase reporter plasmids (Promega Corp, Madison, WI) were used for transient transfections. The pCSE-PGL_3 plasmid containing 3.5-kb CSE promoter (−3,498 ∼ +18) was a kind gift from Dr. I.Ishii (Gunma University Graduate School of Medicine, Gunma, Japan) [41]. Transient transfections were performed using Sofast™ (Sunma Biotech) Cationic Polymer Transfection Reagent according to the manufacturer's manual. Briefly, 1 day before transfection, RAW264.7 cells were seeded and fed with DMEM containing 10% CD-FBS in 48-well plates. Each well was then transfected with 0.1 μg DNA and 10 ng control DNA (pRL-TK-Renilla-luciferase vector, Promega). Ten hours later, culture media were changed, and cells were treated with various reagents as indicated. Dexamethasone and RU38,486 were added to the treatment media as stock solutions in absolute ethanol. Control media contained the same concentration of vehicle (0.01% vol/vol). Luciferase assays were carried out 12 h later using the dual luciferase assay kit (Promega). Relative luciferase activity is presented as firefly luciferase values normalized to Renilla luciferase activity.

MTT assay

Cell viability was assessed by MTT assay. The assay depends on the reduction of the tetrazolium salt MTT by functional mitochondria to formazan [42]. Cells were seeded, cultured and treated as described above. MTT was added at the last 2 h of treatment. After a 2-h incubation of the cells with MTT at 37°C, cells were lysed with dimethyl sulphoxide and the formazan crystals solubilized. Absorbance was read at 550 nm using a spectrophotometric microplate reader (Bio-Rad).

Statistical analysis

Data were expressed as means ± SEM. Statistical significance was estimated by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. A P value <0.05 was considered significant.

Results

Expression of H_2S synthetic enzymes and H_2S synthetic activity in macrophages

RT-PCR analysis showed that CSE but not CBS mRNA was detected in both primary and RAW 264.7 macrophages (Fig. 1a). Western blot analysis showed that CSE but not CBS protein was detected in both primary and RAW 264.7 macrophages. We detected a single protein band of approximately 43 kDa corresponding to CSE protein in primary macrophages and RAW 264.7 cells (Fig. 1b).

The ability of primary macrophages and RAW 264.7 cells to synthesize H_2S from added L-cysteine is demonstrated in Fig. 1c. The initial real-time H_2S production rate observed in primary and RAW 264.7 macrophage

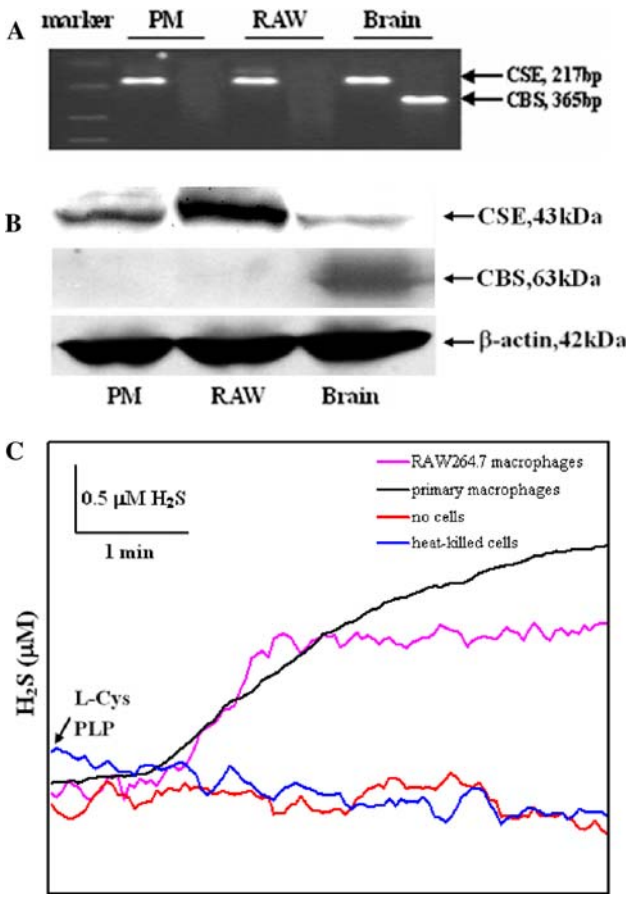

Fig. 1 Expression of H_2S synthetic enzymes and H_2S synthetic activity in macrophages. **a** PCR analysis of CSE and CBS mRNA in macrophages and brain. **b** Western blot analysis showing protein expression of CSE and CBS in macrophages and brain. **c** Representative traces of H_2S production in suspensions of primary and RAW 264.7 macrophages. H_2S production was initiated by the addition of L-cysteine and PLP. There were two control traces of buffered solutions without cells or with heat-killed cells containing L-cysteine and PLP, indicating no spontaneous H_2S production. *PM* primary peritoneal macrophages, *RAW* RAW 264.7 macrophages, *L-Cys* L-cysteine

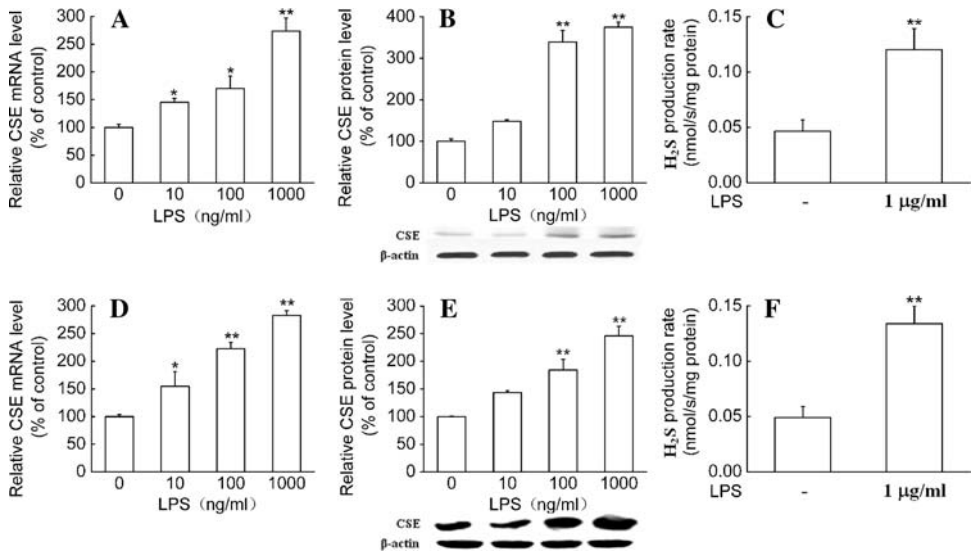

Fig. 2 LPS increased CSE expression and H_2S synthetic activity in macrophages. Primary (**a**–**c**) and RAW 264.7 (**d**–**f**) macrophages were stimulated with LPS at the indicated doses for 6 h (**a**, **d**) or 24 h (**b**, **c**, **e**, **f**), respectively. Quantitative real-time RT-PCR, Western blot analysis and real-time H_2S production measurement were used to determine CSE mRNA expression, protein expression and H_2S synthetic activity in macrophages. Results of Western blot were quantified by scanning densitometry of blots. H_2S production rates were calculated as described in "Materials and Methods." Data were expressed as mean percentage of control ± SEM for CSE mRNA and protein expression ($n = 3$), and for H_2S production rates ($n = 4$). $^*P < 0.05$; $^{**}P < 0.01$ compared with vehicle control

suspensions after addition of L-cysteine and PLP was 0.04653 ± 0.01023 and 0.04882 ± 0.0998 nmol s^{-1} mg protein^{-1} ($n = 4$), respectively.

LPS enhances CSE expression and H_2S synthetic activity in macrophages

Treatment of primary macrophages with increasing concentrations of LPS (10 ng/ml–1 μg/ml) for 6 h or 24 h resulted in increases in CSE mRNA or protein expression in a dose-dependent manner, respectively. Maximal effect was obtained at a concentration of 1 μg/ml, which caused about 2.5-fold increases in CSE mRNA and protein levels. In the meantime, the initial real-time H_2S production rate was also significantly increased in LPS-treated macrophages compared to control (Fig. 2a–c).

In RAW 264.7 cells, LPS also dose-dependently increased CSE mRNA and protein expression. Maximal effect of LPS was achieved at a concentration of 1 μg/ml. LPS also significantly promoted the initial real-time H_2S production rate in RAW264.7 macrophages (Fig. 2d–f).

Endogenous H_2S inhibits NO production in LPS-stimulated macrophages

The study of Oh et al. [29] suggested that, in RAW 264.7 cells, one of the attractive functions of H_2S was inhibition of NO production. In order to investigate if endogenous H_2S has such a function in primary macrophages, we firstly observed the effects of L-cysteine, the precursor of H_2S, and PAG, the CSE inhibitor, on NO production in cultured peritoneal macrophages in the presence of LPS. As shown in Fig. 3, treatment with L-cysteine (1 mmol/l) significantly reduced the expression of inducible NO synthase (iNOS), the predominant NOS in macrophages, as well as NO production in a 24-h treatment period. PAG (1 mmol/l) treatment significantly blocked the L-cysteine-induced inhibition of NO production and iNOS expression in LPS-treated macrophages. Treatment of cells with an H_2S donor, NaHS (200 μmol/l), for 24 h resulted in significant decreases in iNOS expression and NO production in the presence of LPS.

Next, by using genetic approaches including both siRNA and overexpression of CSE, we further confirmed whether endogenous H_2S-mediated inhibition of NO production was specifically dependent on CSE. The CSE siRNA and CSE gene were stably transfected into RAW 264.7 macrophages, thus CSE-knockdown (RAW-mCSE siRNA) and CSE-overexpression (RAW-EGFP-mCSE) RAW264.7 cell lines were established, respectively. Cells were then stimulated with LPS (1 μg/ml) for 24 h. As shown Fig. 4, CSE siRNA resulted in approximately 40% decreases in the H_2S production rate and significantly increased LPS-induced NO production and iNOS expression compared to control siRNA. In contrast, Fig. 5 demonstrated that CSE overexpression resulted in a more than four-fold increase in the H_2S production rate and significantly decreased LPS-induced NO production and iNOS expression compared to the control.

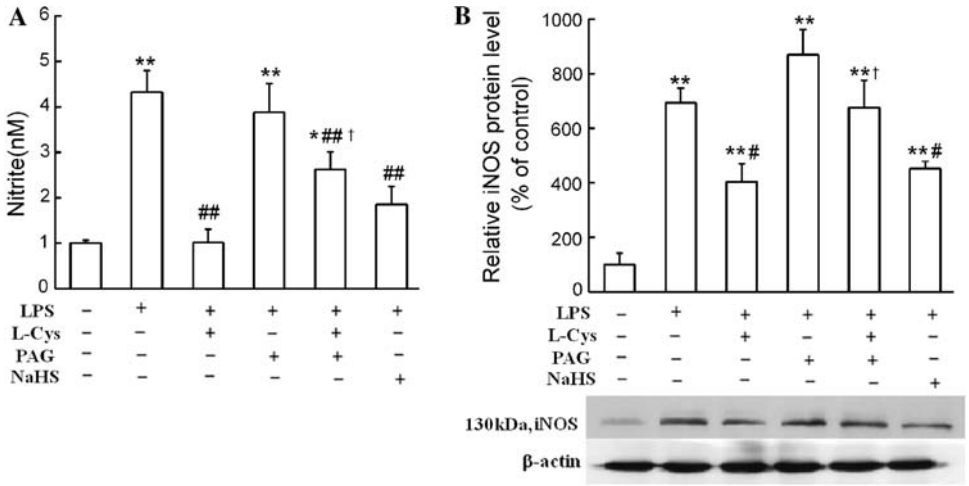

Fig. 3 H_2S inhibited NO production and iNOS expression in LPS-stimulated macrophages. Primary macrophages were treated with 1 mmol/l L-cysteine, 1 mmol/l PAG, or 200 μmol/l NaHS for 24 h in the absence or presence of LPS (1 μg/ml). NO production (**a**) and iNOS protein expression (**b**) were determined as described in "Materials and Methods." Data were expressed as mean ± SEM for NO production ($n = 4$) and mean percentage of control ± SEM for iNOS protein expression ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$ compared with vehicle control; $\#P < 0.05$; $\#\#P < 0.01$ compared with LPS; $\dagger P < 0.05$ compared with LPS plus L-Cys. *L-Cys* L-cysteine

Dexamethasone suppresses H_2S production in LPS-treated macrophages

To determine whether glucocorticoid modulates H_2S production in macrophages, the effects of a potent synthetic glucocorticoid, dexamethasone, on the CSE expression and H_2S production rate in primary and RAW 264.7 macrophages were examined.

In peritoneal macrophages, dexamethasone (10^{-9}–10^{-6} mol/l) significantly inhibited LPS (1 μg/ml)-induced CSE expression at both the mRNA and protein levels. Maximal inhibition of dexamethasone occurred at a concentration of 10^{-6} mol/l, which caused an about 85% decrease in CSE mRNA and 95% decrease in CSE protein levels compared to cells treated with LPS alone (Fig. 6a, b). Consistently, dexamethasone (10^{-7} mol/l) treatment significantly decreased LPS-induced H_2S production rate (Fig.6d). These effects were reversed by RU38,486, a glucocorticoid receptor antagonist (Fig. 6c, d).

In RAW 264.7 cells, it was also shown that dexamethasone treatment decreased CSE mRNA and protein levels as well as the H_2S production rate in the presence of LPS (1 μg/ml) (Fig. 7a–d).

Dexamethasone suppresses NO production in LPS-treated macrophages

We had shown above that decreased endogenous H_2S production resulted in an increase in NO production, and dexamethasone inhibited H_2S production in LPS-treated

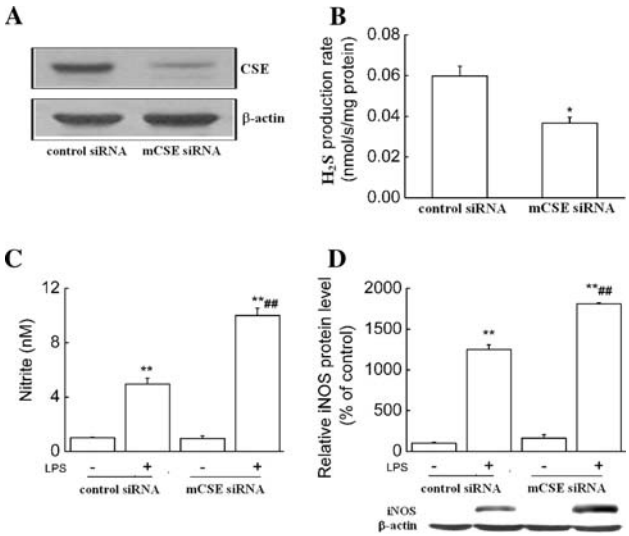

Fig. 4 CSE siRNA increased LPS-induced NO production and iNOS expression. RAW264.7 macrophages were separately transfected with pRNAT-U6.1/Neo-mCSE and pRNAT-U6.1/Neo-Control vectors. Stable CSE-knockdown RAW264.7 cells were selected and maintained in medium containing G418 and named "RAW-mCSE siRNA" ("RAW-control siRNA" as control). Western blot analysis (**a**) and real-time H_2S production measurement (**b**) were used to determine CSE protein expression and H_2S synthetic activity in CSE-knockdown RAW264.7 cells. After stimulation with LPS (1 μg/ml) for 24 h, NO production (**c**) and iNOS protein expression (**d**) were determined as described in "Materials and Methods." Data were expressed as mean ± SEM for H_2S production rates ($n = 3$) and NO production ($n = 4$), and mean percentage of control ± SEM for iNOS protein expression ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$ compared with "RAW-control siRNA"; $\#\#P < 0.01$ compared with "RAW-control siRNA" stimulated with LPS. Control siRNA: RAW-control siRNA; mCSE siRNA: RAW-mCSE siRNA

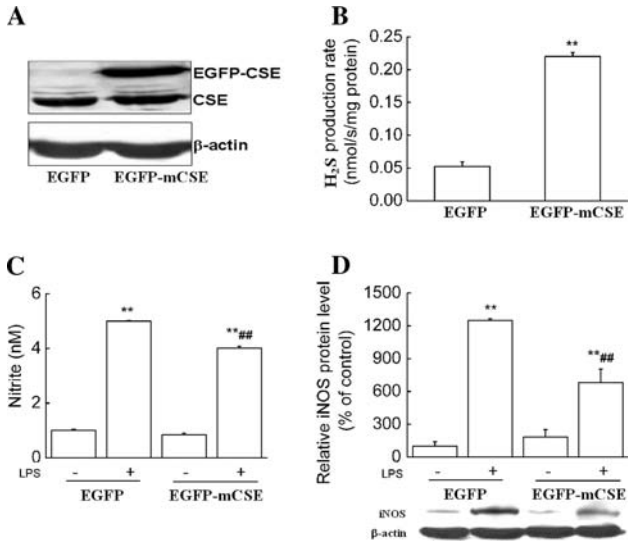

Fig. 5 CSE overexpression decreased LPS-induced NO production and iNOS expression. RAW264.7 macrophages were separately transfected with pEGFP-N3 and pEGFP-mCSE vectors. Stable CSE-overexpression RAW264.7 cells were selected and maintained in medium containing G418 and named "RAW-EGFP-mCSE" ("RAW-EGFP" as control). Western blot analysis (**a**) and real-time H_2S production measurement (**b**) were used to determine CSE protein expression and H_2S synthetic activity in CSE-overexpression RAW264.7 cells. After stimulation with LPS (1 μg/ml) for 24 h, NO production (**c**) and iNOS protein expression (**d**) were determined as described in "Materials and Methods." Data were expressed as mean ± SEM for H_2S production rates ($n = 3$) and NO production ($n = 4$), and mean percentage of control ± SEM for iNOS protein expression ($n = 3$). $^{**}P < 0.01$ compared with "RAW-EGFP"; $^{\#\#}P < 0.01$ compared with "RAW-EGFP" stimulated with LPS. EGFP: RAW-EGFP; EGFP-mCSE: RAW-EGFP-mCSE

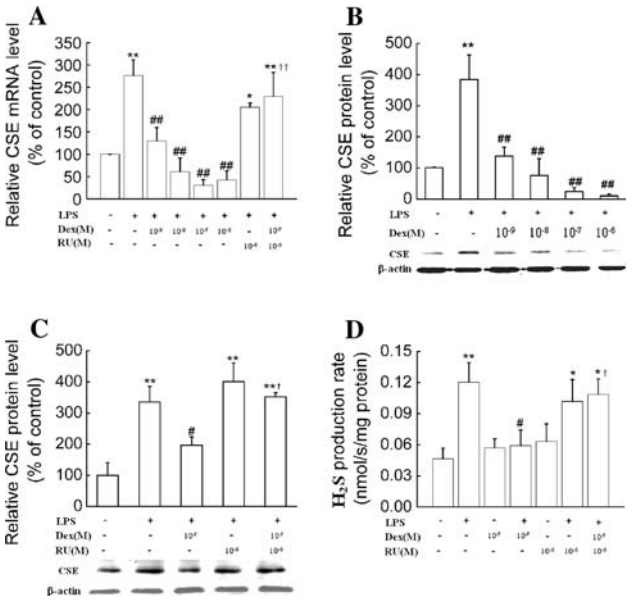

Fig. 6 Dexamethasone suppressed CSE expression and H_2S production in primary macrophages. Primary macrophages were treated with dexamethasone at the indicated doses in the absence or presence of RU38,486 (10^{-6} mol/l) and LPS (1 μg/ml) for 6 h (**a**) or 24 h (**b–d**). CSE mRNA (**a**), protein expression (**b**, **c**) as well as H_2S production rates (**d**) were determined as described in "Materials and Methods." Data were presented as mean percentage of control ± SEM for **a–c** ($n = 3$) and mean ± SEM for H_2S production rates ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with LPS; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with LPS plus dexamethasone. *Dex* dexamethasone, *RU* RU38,486

macrophages. Early studies have demonstrated that dexamethasone inhibits NO production and iNOS expression in macrophage cell lines treated with LPS [43]. Thus, it would be of interest to understand iNOS expression and NO production in primary macrophages treated with dexamethasone. Our results showed that NO production and iNOS expression were significantly decreased in primary macrophages treated with LPS plus dexamethasone compared to cells treated with LPS alone. These effects of dexamethasone were also reversed by RU38,486 (Fig. 8).

To determine whether dexamethasone treatments were detrimental to cells, cell growth and survival were examined. It was found that treatment of cells with dexamethasone for 24 h had no significant effect on cell growth and viability (data not shown).

The role of NO in dexamethasone inhibition of CSE expression and H_2S production in LPS-treated macrophages

In order to explore if the effect of dexamethasone on H_2S production is associated with NO production in LPS-treated macrophages, we firstly investigated the effects of NO precursor and NOS inhibitor on CSE expression and H_2S production. Our results showed that L-arginine (0.1–1 mmol/l) dose-dependently enhanced CSE mRNA and protein expression in LPS-treated primary macrophages. Significant effects occurred at concentrations of 0.5 mmol/l and 1 mmol/l. Consistently, L-arginine (1 mmol/l) treatment significantly increased the LPS-induced H_2S production rate (Fig. 9a–c). L-NAME (0.25–1 mmol/l), a NOS inhibitor, decreased CSE mRNA and protein levels in a dose-dependent manner. The LPS-induced H_2S production rate was significantly decreased by L-NAME (1 mmol/l) treatment (Fig. 9d–f). These results suggest that NO is an important factor for CSE expression and H_2S production.

We then observed the effects of dexamethasone on LPS-induced CSE expression and H_2S production in the presence of NOS inhibitor. As shown in Fig. 10, dexamethasone (10^{-9}–10^{-6} mol/l) treatment in the presence of L-NAME (1 mmol/l) significantly decreased LPS-induced expression of CSE mRNA and protein compared with L-NAME. Dexamethasone (10^{-7} mol/l) plus L-NAME (1 mmol/l) also significantly decreased LPS-induced H_2S production rates compared with the cells treated with L-NAME (1 mmol/l). These results suggest that dexamethasone not only indirectly

decreases CSE expression and H_2S production by inhibiting NO production, but also may directly suppress CSE expression in LPS-treated macrophages.

Dexamethasone reduces CSE mRNA stability in primary macrophages

As a first step in elucidating the molecular mechanisms by which dexamethasone decreased CSE mRNA, we measured the rate of CSE mRNA degradation. An inhibitor of mRNA synthesis, actinomycin D (1 μg/ml) was added to the cells 6 h after LPS or LPS plus dexamethasone treatment. Cells were harvested at time points of 0, 4 and 8 h for primary macrophages and 0, 4, 8 and 12 h for RAW 264.7 cells after the addition of actinomycin D.

In primary macrophages, dexamethasone treatment significantly decreased the half-life of CSE mRNA in LPS-treated cells. The half-life of CSE mRNA after LPS treatment was 2.52 ± 0.26 h, and dexamethasone reduced it to 1.67 ± 0.04 h. (***$P < 0.05$, Fig. 11a). In RAW 264.7 cells (Fig. 11b), the half-life of CSE mRNA was 7.60 ± 0.61 h in LPS-treated cells and 7.59 ± 0.46 h in LPS plus dexmethasone-treated cells. There was no statistical difference between these two groups. These results suggested that dexamethasone reduced CSE mRNA stability in primary macrophages, but not in RAW 264.7 cells.

Dexamethasone reduced CSE transcription in RAW 264.7 macrophages

To determine the effects of dexamethasone on the rate of CSE gene transcription in RAW 264.7 cells, we transfected the pCSE-PGL_3 plasmid into RAW 264.7 cells and examined the CSE promoter activity. As shown in Fig. 12, dexamethasone (10^{-8}–10^{-6} mol/l) treatment significantly decreased the transcriptional activity of CSE genes compared with LPS alone, and these effects of dexamethasone were also reversed by RU38,486, suggesting that dexamethasone suppressed CSE expression by repressing CSE gene transcription in RAW264.7 macrophages.

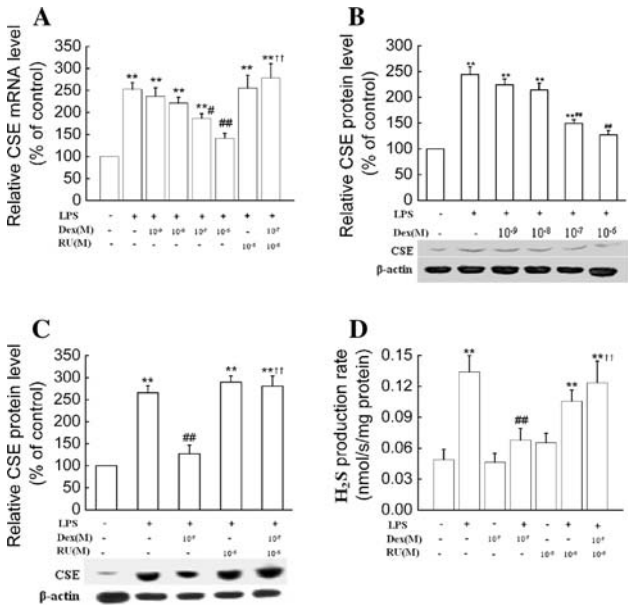

Fig. 7 Dexamethasone suppressed CSE expression and H_2S production in RAW264.7 macrophages. RAW264.7 macrophages were treated with dexamethasone at the indicated doses in the absence or presence of RU38,486 (10^{-6} mol/l) and LPS (1 μg/ml) for 6 h (**a**) or 24 h (**b**–**d**). CSE mRNA (**a**), protein expression (**b**, **c**) as well as H_2S production rates (**d**) were determined as described in "Materials and Methods." Data were presented as mean percentage of control ± SEM for **a**–**c** ($n = 3$) and mean ± SEM for H_2S production rates ($n = 4$). **$P < 0.01$ compared with control; #$P < 0.05$, ##$P < 0.01$ compared with LPS; ††$P < 0.01$ compared with LPS plus dexamethasone. *Dex* dexamethasone, *RU* RU38,486

Discussion

Macrophages are shown to produce numerous mediators including the gaseous mediators NO and CO in response to inflammatory stimuli (e.g., LPS) [27, 28]. In this study, we showed that, for the first time, primary macrophages had the capacity to produce H_2S, the production rate of which

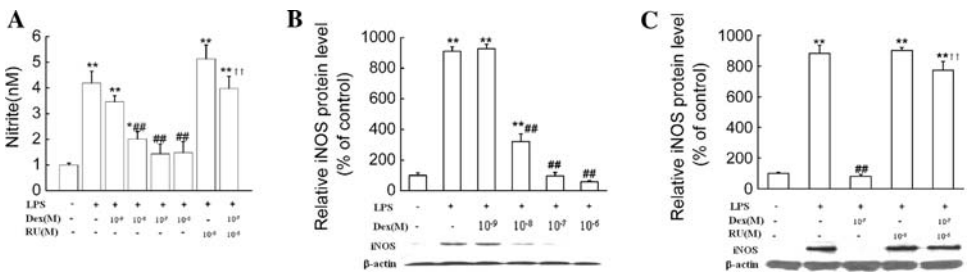

Fig. 8 Dexamethasone suppressed NO production and iNOS expression in primary macrophages. Primary macrophages were treated with dexamethasone at the indicated doses in the absence or presence of RU38,486 (10^{-6} mol/l) and LPS (1 μg/ml) for 24 h. NO production (**a**) and iNOS expression (**b**, **c**) were determined as described in "Materials and Methods." Data were presented as mean ± SEM for NO production ($n = 4$) and mean percentage of control ± SEM for iNOS expression ($n = 3$). *$P < 0.05$; **$P < 0.01$ compared with control; ##$P < 0.01$ compared with LPS; ††$P < 0.01$ compared with LPS plus dexamethasone. *Dex* dexamethasone, *RU* RU38,486

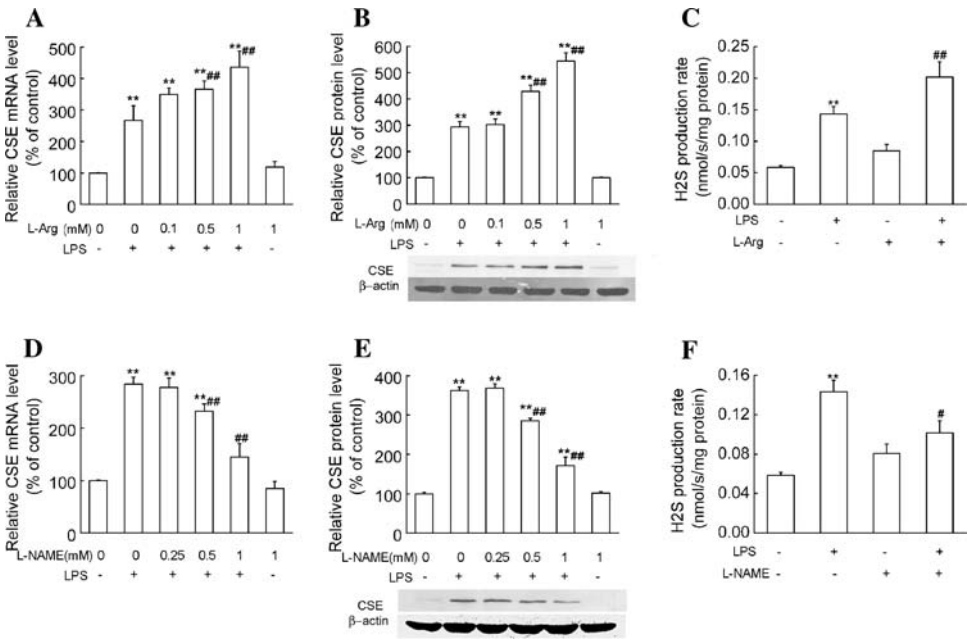

Fig. 9 Effects of NO precursor and NOS inhibitor on CSE expression and H_2S production in primary macrophages stimulated with LPS. Primary macrophages were treated with NO precursor L-arginine (**a**–**c**) or NOS inhibitor L-NAME (**d**–**f**) at the indicated doses in the absence or presence of LPS (1 μg/ml) for 6 h (**a**, **d**) or 24 h (**b**, **c**, **e**, **f**), respectively. Quantitative real-time RT-PCR, Western blot analysis and real-time H_2S production measurement were used to determine CSE mRNA expression, protein expression and H_2S synthetic activity in macrophages. Results of Western blot were quantified by scanning densitometry of blots. H_2S production rates were calculated as described in "Materials and Methods." Data were expressed as mean percentage of control ± SEM for CSE mRNA and protein expression ($n = 3$), and for H_2S production rates ($n = 4$). $^{**}P < 0.01$ compared with vehicle control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with LPS. *L-arg* L-arginine

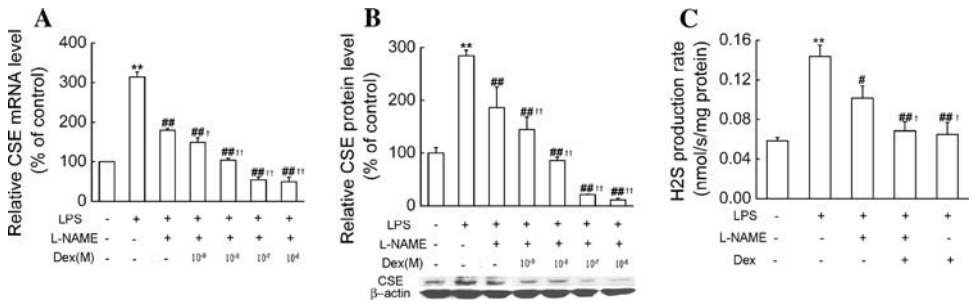

Fig. 10 Effects of dexamethasone on LPS-induced CSE expression and H_2S production in the presence of NOS inhibitor in primary macrophages. Primary macrophages were treated with dexamethasone at the indicated doses in the absence or presence of L-NAME (1 mmol/l) and LPS (1 μg/ml) for 6 h (**a**) or 24 h (**b**–**c**). CSE mRNA (**a**), protein expression (**b**) as well as H_2S production rates (**c**) were determined as described in "Materials and Methods." Data were presented as mean percentage of control ± SEM for **a**–**b** ($n = 3$) and mean ± SEM for H_2S production rates ($n = 4$). $^{**}P < 0.01$ compared with control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with LPS; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with LPS plus L-NAME. *Dex* dexamethasone

could be further enhanced by LPS treatment; endogenous H_2S was capable of inhibiting NO production locally in macrophages.

H_2S is formed in mammalian cells mainly by CBS and CSE. CBS and CSE are widely distributed in tissues, although a degree of tissue specificity is apparent. CBS is more abundant in brain, whereas CSE activity is most notable in peripheral tissues such as blood vessels [44]. In liver and kidney, both CBS and CSE have been identified [45]. The present study showed that primary and RAW 264.7 macrophages expressed CSE but not CBS and were capable of producing H_2S through CSE. Earlier studies have shown marked increases in the plasma H_2S level and H_2S production in tissues including liver and kidney during several inflammatory conditions [6, 7, 9, 10]. In the present experiments, the expression of CSE was significantly enhanced by LPS, the major component of the outer membrane of gram-negative bacteria, in both primary and

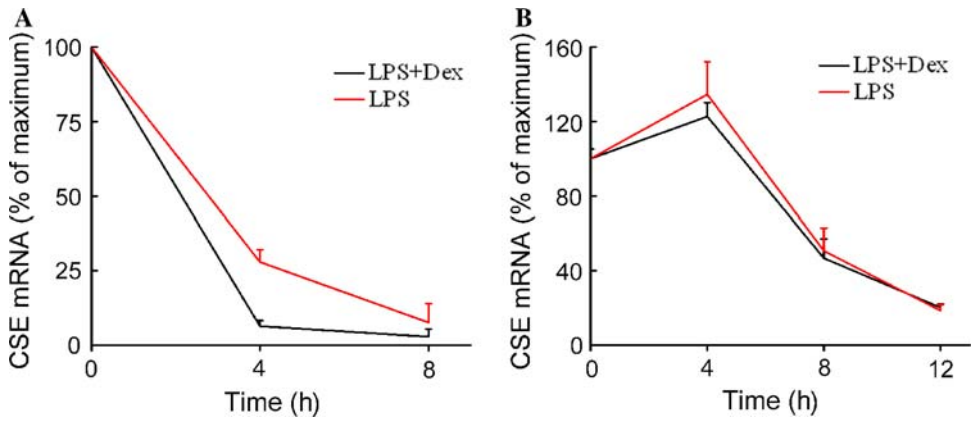

Fig. 11 Effects of dexamethasone on CSE mRNA stability in macrophages stimulated with LPS. Primary (**a**) and RAW 264.7 (**b**) macrophages were stimulated with LPS (1 μg/ml) in the absence or presence of dexamethasone (10^{-7} mol/l) as indicated. Actinomycin D (1 μg/ml) was added to the cells after 6 h of incubation. Cells treated with vehicle were used as the maximum point. Cells were harvested for RNA extraction after the incubation time indicated after actinomycin D addition, and CSE and GAPDH mRNA were detected by quantitative real-time RT-PCR. Each data point was expressed as mean percentage of the maximum determined at time zero ± SEM ($n = 4$). *Dex* dexamethasone

RAW 264.7 macrophages, and consistently H_2S synthesis was also elevated. This suggests that macrophages may be one of the H_2S-producing sources during gram-negative bacteria-induced inflammation.

There are numerous conflicting data regarding the effects of H_2S on inflammation. For example, CSE inhibitor has been reported both to exacerbate [13] and to reduce [46] carrageenan-induced paw edema. Both H_2S donors and CSE inhibitors exhibit anti-inflammatory activities such as inhibiting leukocyte/endothelium adhesion [13, 47]. To date, much of our knowledge of the biological effects of H_2S comes from the use of inhibitors of H_2S synthases, H_2S donors or H_2S precursor. However, the specificity of these reagents is doubted. For instance, CSE inhibitor PAG targets the pyridoxal 5′-phosphate-binding site of CSE and, as such, may affect other pyridoxal 5′-phosphate-dependent enzymes as well [48]. Thus, interpretations of results from experiments using those above-mentioned reagents must be made with caution. To avoid such limitations in specificity of those reagents, we used both CSE and CSE-siRNA expression vector to specifically increase and decrease CSE expression, respectively. In consistence with the results obtained by using L-cysteine, we demonstrated that CSE siRNA significantly increased, whereas CSE overexpression decreased LPS-induced iNOS expression and NO production. NO is well-recognized as one of the pro-inflammatory mediators, and NO production is markedly increased during various inflammatory conditions such as endoxemia [49]. Our results showed that endogenous H_2S produced locally in macrophages has a tonic inhibitory effect on NO production in the presence of LPS. Thus, this suggests that H_2S might exert anti-inflammatory effects by inhibiting

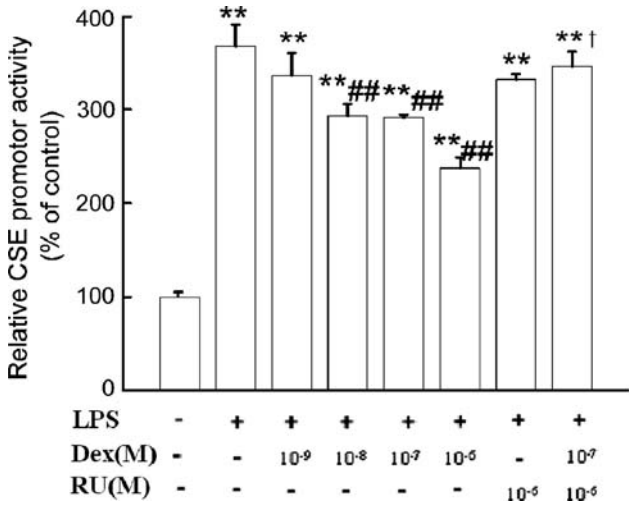

Fig. 12 Effects of dexamethasone on CSE promoter activity in RAW264.7 macrophages. RAW264.7 cells were treated with dexamethasone at the indicated doses in the absence or presence of RU38,486 (10^{-6} mol/l) and LPS (1 μg/ml) for 12 h. CSE promoter activity was assayed as described in "Materials and Methods." Data were presented as mean percentage of control ± SEM for four independent experiments. $^{**}P < 0.01$ compared with control; $\#\#P < 0.01$ compared with LPS; $\dagger P < 0.05$ compared with LPS plus dexamethasone. *Dex* dexamethasone, *RU* RU38,486

NO production in macrophages during LPS-induced inflammation.

Several studies have demonstrated the impact of NO on H_2S production. Zhao et al. [5] reported that, in cultured smooth muscle cells, NO increases CSE mRNA expression and H_2S production. Zhong et al. [50] found that inhibition of NO production caused a decrease in H_2S production and CSE activity in artery in vivo. However, a study of Anuar and coworkers suggested that NO can inhibit H_2S

production by showing that administration of NO donor to LPS-treated rat resulted in a decrease in the formation of H_2S and CSE expression in liver [51]. Our findings that NOS inhibitor decreased whereas NO precursor increased H_2S production and CSE expression in LPS-treated macrophages suggest that NO is an important endogenous stimulus in the formation of H_2S during LPS-induced inflammation.

GCs are known to exert their anti-inflammatory effects by inhibiting the expression and production of various pro-inflammatory mediators, such as TNF-α, IL-1 and NO, as well as stimulating the expression of genes coding for anti-inflammatory proteins, such as lipocortin-1 and interleukin-10 [17]. Our findings that dexamethasone inhibited NO production through iNOS expression are consistent with previous studies [43, 52]. Interestingly, our present study also found that decreased H_2S production resulted in increases in NO production and iNOS expression, and dexamethasone reduced H_2S production. This would suggest that dexamethasone inhibition of NO production and iNOS expression in LPS-treated macrophages was not related to H_2S. As mentioned above, the present study found that NO had a stimulatory effect on CSE expression and H_2S production in LPS-treated macrophages. Moreover, when NO production was inhibited by NOS inhibitor, L-NAME, dexamethasone could intensively inhibit CSE expression and H_2S production compared to L-NAME. This suggests that dexamethasone may directly inhibit CSE expression and H_2S production, besides the NO-dependent way.

More recently, Li et al. proposed that inhibition of H_2S production in neutrophils contributes to the anti-inflammatory effect of dexamethasone in endotoxic shock [33]. However, H_2S has been indicated to have anti-inflammatory effects in previous investigations [13, 48, 53]. The present study also indicates that H_2S might play an anti-inflammation role by inhibiting NO production in macrophages. In this case, our findings that GCs inhibit H_2S and NO production provide evidence that GCs coordinate the production of pro- and anti-inflammatory mediators during inflammation. Recently, Van Molle and Libert [20] proposed that GCs could balance between pro- and anti-inflammatory mediators and control their own strength by inducing the pro-inflammatory cytokine MIF.

The classic effects of glucocorticoids are mediated through GR. RU38,486 is a steroid that competitively binds to GR and inhibits the effect of glucocorticoids [54]. In the presence of RU38,486, dexamethasone did not inhibit CSE expression and the H_2S production rate in LPS-treated cells, indicating that suppressed H_2S production is a GR-mediated process. Glucocorticoid-GR complexes are able to activate or inhibit gene transcription [55]. In the present study, we also found that dexamethasone repressed CSE transcription in LPS-treated RAW 264.7 cells.

In addition to its effects on gene transcription, GCs have been shown to regulate the mRNA stability of some genes. Korhonen et al. [43] showed that dexamethasone decreases LPS-induced iNOS expression by destabilizing iNOS mRNA in J774 macrophages. The study of Walker and coworkers suggested that dexamethasone inhibits iNOS expression by destabilizing mRNA and repressing gene transcription interferon-γ-stimulated macrophages [52]. We found that dexamethasone reduced CSE mRNA stability in primary macrophages, but did not affect that in RAW 264.7 cells. In RAW 264.7 cells, dexamethasone suppressed CSE promoter activity. These findings suggest that dexamethasone inhibition of CSE expression takes place through different mechanisms in primary macrophages and macrophage cell lines. On the other hand, these data also suggest that the mechanism of action of glucocorticoid on CSE expression might be related to cell type.

In conclusion, this study demonstrated that macrophages expressed CSE and had the capacity to produce H_2S through CSE. Endogenous H_2S had a tonic inhibitory effect on NO production in macrophages. Dexamethasone suppressed the LPS-induced H_2S production rate through regulating CSE expression in macrophages. Dexamethasone could also inhibit CSE expression and H_2S production by inhibiting NO production because endogenous NO enhanced CSE expression and dexamethasone inhibited NO production in LPS-treated macrophages. Destabilizing mRNA was involved in dexamethasone inhibition of LPS-induced CSE expression and H_2S production in primary macrophages. Repression of gene transcription was responsible for dexamethasone inhibition of CSE expression and H_2S production in RAW 264.7 cells. These data expanded our knowledge of the mechanisms of LPS-induced inflammatory processes as well as GCs' regulation of inflammation.

Acknowledgments The authors wish to thank Dr. I.Ishii, Gunma University Graduate School of Medicine, Gunma, Japan, for his gift of pCSE-PGL_3 plasmid. This work was supported by the National Natural Science Foundation of China, grant nos. 30670815 and 30770846, and the Science and Technology Commission of Shanghai Municipals (09XD1405600).

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