

Effect of endotoxemia in mice genetically deficient in cystathionine- γ -lyase, cystathionine- β -synthase or 3-mercaptopyruvate sulfurtransferase

AKBAR AHMAD, DOMOKOS GERÖ, GABOR OLAH and CSABA SZABO

Department of Anesthesiology, University of Texas Medical Branch, Galveston, TX 77555-1102, USA

Received July 14, 2016; Accepted September 14, 2016

DOI: 10.3892/ijmm.2016.2771

Abstract. Hydrogen sulfide (H_2S) has been proposed to exert pro- as well as anti-inflammatory effects in various models of critical illness. In this study, we compared bacterial lipopolysaccharide (LPS)-induced changes in inflammatory mediator production, indices of multiple organ injury and survival in wild-type (WT) mice and in mice with reduced expression of one of the three H_2S -producing enzymes, cystathionine- γ -lyase (CSE), cystathionine- β -synthase (CBS) or 3-mercaptopyruvate sulfurtransferase (3MST). Mice were injected intraperitoneally (i.p.) with LPS (10 mg/kg). After 6 h, the animals were sacrificed, blood and organs were collected and the following parameters were evaluated: blood urea nitrogen (BUN) levels in blood, myeloperoxidase (MPO) and malondialdehyde (MDA) in the lung, cytokine levels in plasma and the expression of the three H_2S -producing enzymes (CBS, CSE and 3MST) in the spleen, lung, liver and kidney. LPS induced a tissue-dependent upregulation of some of the H_2S -producing enzymes in WT mice (upregulation of CBS in the spleen, upregulation of 3MST in the liver and upregulation of CBS, CSE and 3MST in the lung). Moreover, LPS impaired glomerular function, as evidenced by increased BUN levels. Renal impairment was comparable in the CSE^{-/-} and Δ 3MST mice after LPS challenge; however, it was attenuated in the CBS^{+/-} mice. MPO levels (an index of neutrophil infiltration) and MDA levels (an index of oxidative stress) in lung homogenates were significantly increased in response to LPS; these effects were similar in the WT, CBS^{+/-}, CSE^{-/-} and Δ 3MST mice;

however, the MDA levels tended to be lower in the CBS^{+/-} and CSE^{-/-} mice. LPS induced significant increases in the plasma levels of multiple cytokines [tumor necrosis factor (TNF) α , interleukin (IL)-1 β , IL-6, IL-10, IL-12 and interferon (IFN) γ] in plasma; TNF α , IL-10 and IL-12 levels tended to be lower in all three groups of animals expressing lower levels of H_2S -producing enzymes. The survival rates after the LPS challenge did not show any significant differences between the four animal groups tested. Thus, the findings of this study indicate that a deficiency in 3MST does not significantly affect endotoxemia, while a deficiency in CBS or CSE slightly ameliorates the outcome of LPS-induced endotoxemia *in vivo*.

Introduction

Three major hydrogen sulfide (H_2S)-producing enzymes have been identified: cystathionine- γ -lyase (CSE), cystathionine- β -synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3MST) (1-10). H_2S is known to regulate a multitude of physiological and pathophysiological functions in the vascular, immune and nervous system (1-10).

The role of H_2S in various forms of critical illness has been a subject of intensive investigations over the past decade. Some studies have demonstrated the therapeutic effect of H_2S donation in various models of circulatory shock (11-16), while others have reported that the pharmacological inhibition of H_2S production (17-21) or the genetic deficiency of H_2S -producing enzymes (22,23) results in beneficial effects.

The aim of the current study was to examine the effect of lipopolysaccharide (LPS)-induced changes in inflammatory mediator production, indices of multiple organ injury and survival in wild-type (WT) mice and in mice with reduced expression of one of the three H_2S -producing enzymes, CSE, CBS or 3MST. We compared the effect of bacterial LPS in WT, CBS heterozygous (CBS^{+/-}), CSE knockout (CSE^{-/-}) or 3MST mutant (Δ 3MST) mice.

Materials and methods

Materials. Unless indicated otherwise, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animals and experimental design. Male WT mice (C57/BL6), CBS heterozygous mice [CBS^{+/-}; Jackson Laboratory, Ben

Correspondence to: Dr Csaba Szabo, Department of Anesthesiology, University of Texas Medical Branch, 601 Harborside Drive, Building 21, Room 4.202D, Galveston, TX 77555-1102, USA
E-mail: szabocsaba@aol.com

Abbreviations: BCA, bicinchoninic acid; BUN, blood urea nitrogen; CBS, cystathionine- β -synthase; CSE, cystathionine- γ -lyase; H_2S , hydrogen sulfide; IL, interleukin; LPS, lipopolysaccharide; MDA, malondialdehyde; MPO, myeloperoxidase; 3MST, 3-mercaptopyruvate sulfurtransferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, standard error of the mean

Key words: hydrogen sulfide, shock, inflammation, nitric oxide

Harbor, ME, USA, as previously described (24)], CSE knockout mice [CSE^{-/-}; a gift from Dr Solomon Snyder, Johns Hopkins University, as previously described (25)] and 3MST mutant mice [Δ 3MST; generated at the Texas A&M University, as previously described (26)] (all 2 months of age) were housed in a light-controlled room with a 12-h light-dark cycle and were allowed *ad libitum* access to food and water. Current studies utilize CBS heterozygous mice, due to the high mortality rate of CBS^{-/-} mice after birth (25). All investigations adhered to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Eighth Edition, 2011) and were performed in accordance with the IACUC, University of Texas Medical Branch, Galveston, TX, USA.

LPS-induced endotoxemia in mice. Mice were randomly allocated into the following groups: i) WT mice + vehicle (n=10); ii) WT mice + LPS [10 mg/kg, intraperitoneally (i.p.)] (n=10); iii) CBS^{+/-} mice + vehicle (n=10); iv) CBS^{+/-} mice + LPS (10 mg/kg, i.p.) (n=10); v) CSE^{-/-} mice + vehicle (n=10); vi) CSE^{-/-} mice + LPS (10 mg/kg, i.p.) (n=10); vii) Δ 3MST mice + vehicle (n=10); and viii) Δ 3MST mice + LPS (10 mg/kg, i.p.) (n=10). The volume of saline (V) administered was equal to the volume of LPS administered. Six hours after the LPS injection the mice were sacrificed by isoflurane inhalation (0.25-3%) followed by opening of the chest and exsanguination by cardiac puncture; blood and tissue samples were then collected for further examinations. This time point was selected based on prior studies showing that at this point LPS-induced cytokine responses are detectable (including those that are released early on); at the same time, multiple organ injury is already significant (27-30); however at this time point, no mortality ensues yet.

Expression of CBS, CSE and 3MST in lung, spleen, liver and kidney samples. The organs were placed in RIPA buffer and sonicated (three times for 10 sec each). The supernatants were preserved and the protein concentration was determined by bicinchoninic acid (BCA) assay. Protein expression was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The supernatant extracts (40 μ g/ μ l) were boiled in equal volumes of loading buffer (150 mM Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 15% β -mercaptoethanol; and 0.01% bromophenol blue) and were electrophoresed on 8-12% polyacrylamide gels. Following electrophoretic separation, the proteins were transferred onto PVDF membranes for western blotting. The membranes were blocked with StartingBlock T20 (TBS) Blocking Buffer (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h. The following primary antibodies were used: CBS, 1:1,000 (GTX628777; GeneTex, Inc., Irvine, CA, USA); CSE, 1:1,000 (12217-1-AF; ProteinTech Group, Inc., Chicago, IL, USA); 3MST, 1:1,000 (HPA001240; Sigma-Aldrich); and actin, 1:5,000 (sc-1616; Cell Signaling Technology, Inc., Danvers, MA, USA). The primary antibodies were incubated overnight at 4°C and the membranes were washed twice in TBST. Secondary horseradish peroxidase-conjugated antibodies [anti-rabbit (7074S), anti-mouse (7076S); Cell Signaling Technology, Inc.] were then applied at a dilution of 1:5,000 for 1 h. Over a 30-min period, the blots were washed twice in TBST, after which they were incubated in

enhanced chemiluminescence reagents (SuperSignal detection kit; Pierce Biotechnology, Inc., Rockford, IL, USA). The band intensity of the original blots was quantified using GeneTools (Syngene; Synoptics, Ltd., Cambridge, UK) and normalized to actin expression.

Assessment of renal dysfunction. At 6 h post-LPS challenge, blood samples were collected via cardiac puncture and were analyzed by using a VetScan analyzer (Abaxis North America, Union City, CA, USA). The ratio of the blood concentration of urea was calculated as an indicator of glomerular function.

Malondialdehyde (MDA) assay. Tissue MDA levels, an index of cellular injury/oxidative stress, were quantified in lung samples using a fluorimetric MDA-Specific Lipid Peroxidation assay kit (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions. The assay is based on the BML-AK171 method in which two molecules of the chromogenic reagent *N*-methyl-2-phenylindole react with one molecule of MDA at 45°C to yield a stable carbocyanine dye with a maximum absorption at 586 nm.

Myeloperoxidase (MPO) assay. MPO activity was measured in lung samples using a commercially available MPO fluorometric detection kit (Enzo Life Sciences). The assay utilizes a non-fluorescent detection reagent, which is oxidized in the presence of hydrogen peroxide and MPO to produce its fluorescent analog. The fluorescence is measured at excitation wavelength of 530-571 nm and emission wavelength of 590-600 nm.

Quantification of plasma cytokine levels. Blood from mice in all groups was collected in K2EDTA blood collection tubes and centrifuged at 4°C for 15 min at 2,000 x g within 30 min of collection. Plasma was isolated, aliquoted and stored at -80°C until use. The EMD Millipore's MILLIPLEX™ MAP Mouse Cytokine Magnetic Bead Panel 1 kit (EMD Millipore, Billerica, MA, USA) was used for the simultaneous quantification of the following analytes: interleukin (IL)-1 β , tumor necrosis factor (TNF) α , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, interferon (IFN) γ , granulocyte-macrophage colony-stimulating factor (GM-CSF) (Merck Millipore, Darmstadt, Germany). Luminex uses a proprietary technique to internally color code microspheres with two fluorescent dyes and to create distinctly colored bead sets of 500 polystyrene microspheres (5.6 μ m) or 80 magnetic microspheres (6.45 μ m), each of which is coated with a specific capture antibody. After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with streptavidin-phycoerythrin conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere. The Luminex instrument acquires and analyzes data using the Luminex xMAP fluorescent detection method and the Luminex xPONENT™ acquisition software (Thermo Fisher Scientific).

Survival analyses. Survival was assessed in the WT, CBS heterozygous (CBS^{+/-}), CSE knockout (CSE^{-/-}) or 3MST mutant (Δ 3MST) mice (n=15 mice in each group) after i.p. injection of LPS (20 mg/kg, i.p.). Mortality of the animals was recorded over a 48-h period.

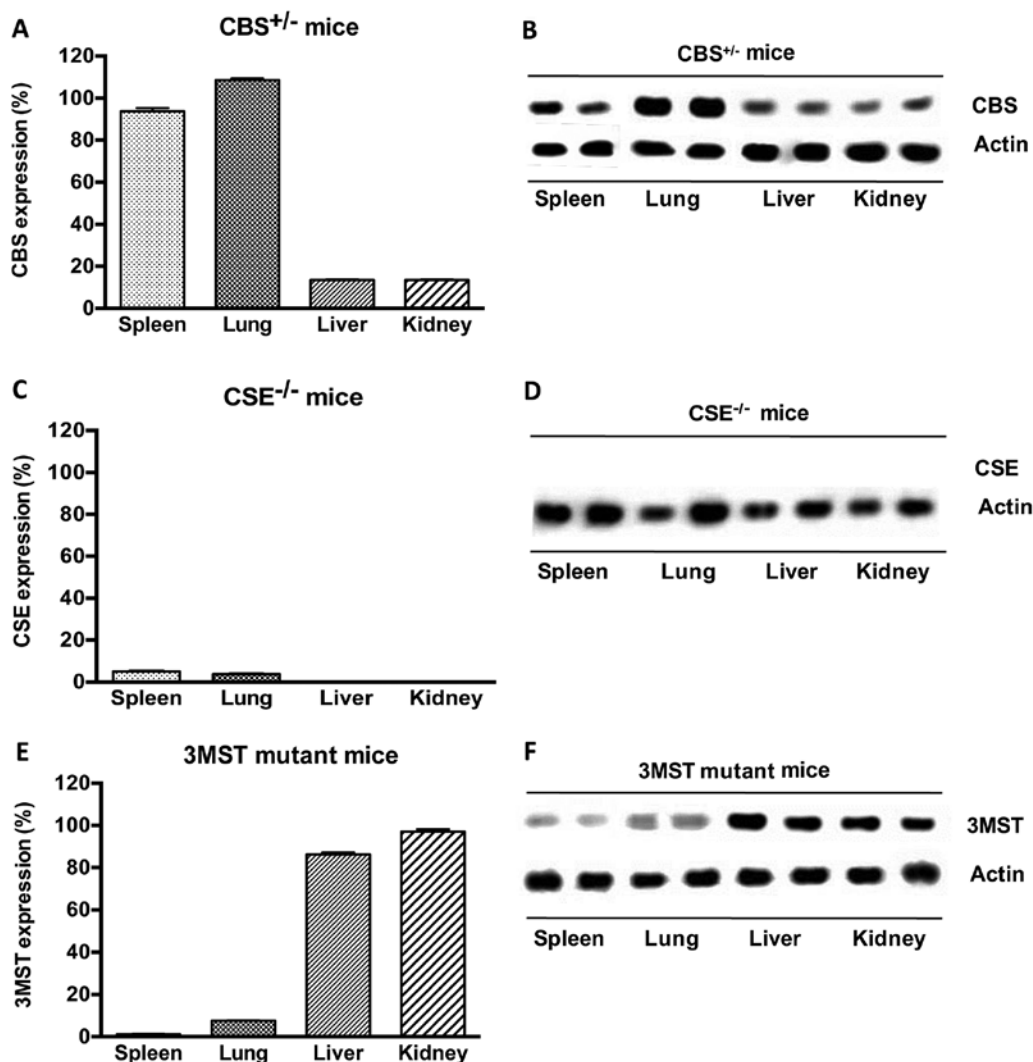


Figure 1. Baseline tissue expression of cystathionine- β -synthase (CBS), cystathionine- γ -lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3MST) in the animals used in the current study. Protein levels of (A and B) CBS, (C and D) CSE and (E and F) 3MST in the spleen, lung, liver and kidney of the CBS heterozygous (CBS^{+/-}), CSE knockout (CSE^{-/-}) and 3MST mutant (Δ 3MST) mice are shown. Enzyme levels were normalized to those of the wild-type control, set as 100%. Please note that CBS^{+/-} mice show reduced CBS expression in the liver and kidney; CSE^{-/-} mice show reduced CSE expression in the spleen, lung, liver and kidney; and Δ 3MST mice show reduced 3MST expression in the spleen and lung. Data are shown as the means \pm standard error of the mean (SEM) of n=5 determinations.

Statistical analysis. All values described in the text and figures are expressed as the means \pm standard error of the mean (SEM) for 'n' observations. The Student's t-test, one- and two-way ANOVA with Tukey's post hoc test were used to detect differences between groups. The Chi-square test was used to compare survival rates. Prism version 5 for Windows (GraphPad Software, Inc., La Jolla, CA, USA) was used. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Changes in the expression of H₂S-producing enzymes in response to LPS. First, the effect of LPS on the expression of the three H₂S-producing enzymes (CBS, CSE and 3MST) was examined in various tissue samples (spleen, lung, liver and kidney) in the control (vehicle-treated) WT, CBS^{+/-}, CSE^{-/-} and Δ 3MST mice. We found the following basal expression of the enzymes (Fig. 1): in CSE^{-/-} mice, CSE protein was absent in

all tissues studied; in CBS^{+/-} mice, CBS levels were markedly suppressed in some tissues (liver, kidney), while they remained unaltered in others (spleen, lung), indicating that in some tissues a single copy of the CBS gene is sufficient to yield physiological amounts of CBS transcripts. In addition, and as previously observed (26), the current strain of Δ 3MST mice exhibited reduced 3MST expression in their spleens and lungs, but not the livers and kidneys. We then examined the effect of LPS challenge on the expression of CBS, CSE and 3MST in WT mice. LPS induced an increase in CBS expression in the spleen and lung; CSE expression increased in the lung and 3MST expression increased in the lung and liver (Fig. 2). These expression patterns were, generally, similar in the WT mice and the genetically modified strains of mice, even though in the CSE^{-/-} mice, the LPS-induced upregulation of CBS occurred in the liver and kidney and in the Δ 3MST mice, it only occurred in the kidney (as opposed to the WT mice, where it occurred in the spleen and the lung). Moreover, in response to LPS, the upregulation of CSE in the CBS^{+/-} mice

Table I. Expression profiles of cystathionine- β -synthase (CBS), cystathionine- γ -lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3MST) at 6 h after the lipopolysaccharide (LPS) (10 mg/kg) injection in wild-type (WT), CBS^{+/-}, CSE^{-/-} and Δ 3MST mice.

	WT (%)	CBS ^{+/-} (%)	CSE ^{-/-} (%)	Δ 3MST (%)
CBS expression				
Spleen	227±29 ^a	168±15 ^a	111±6	107±15
Lung	134±12 ^a	115±6	109±17	120±10
Liver	112±12	19±6	149±11 ^a	107±13
Kidney	112±14	19±5	161±15 ^a	123±9 ^a
CSE expression				
Spleen	116±14	125±15 ^a	0	91±9
Lung	134±19 ^a	92±16	0	120±6 ^a
Liver	92±5	101±6	0	103±22
Kidney	119±1	106±1.6	0	103±12
3MST expression				
Spleen	116±8	125±10 ^a	125±11 ^a	1±1
Lung	177±25 ^a	159±20 ^a	241±14 ^a	9±1
Liver	139±9 ^a	129±12 ^a	111±11	107±13
Kidney	110±13	114±8	9±1	115±13

^aP<0.05 shows significant change compared to baseline control in wild-type mice (which is considered as 100%). Data are shown as the means \pm standard error of the mean (SEM) of n=5 determinations; n=5.

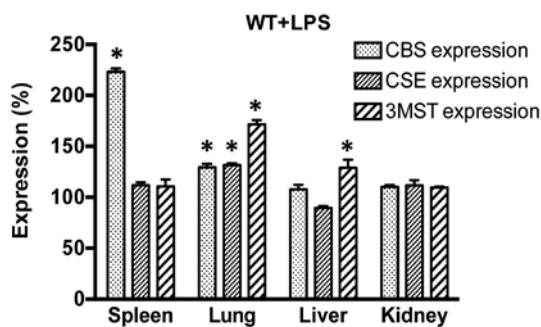


Figure 2. Effect of lipopolysaccharide (LPS) [10 mg/kg, intraperitoneally (i.p.), 6 h] on protein levels of cystathionine- β -synthase (CBS), cystathionine- γ -lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3MST) in the spleen, lung, liver and kidney of wild-type (WT) mice. Expression levels in vehicle-treated mice for each organ are normalized as 100%. Please note that LPS induced an increase in CBS expression in the spleen and lung, an increase in CSE expression in the lung, and an increase in 3MST expression in the lung and liver. Data are shown as the means \pm standard error of the mean (SEM) of n=5 determinations; *p<0.05 shows significant LPS-induced increase in the expression level of the indicated enzyme in the indicated organ.

occurred in the spleen (whereas in the WT mice the largest degree of CSE upregulation occurred in the lung) (Table I).

Effect of CBS^{+/-}, CSE^{-/-} and Δ 3MST on LPS-induced blood urea nitrogen (BUN) plasma levels. LPS administration to all four groups of mice studied (WT, CBS^{+/-}, CSE^{-/-} and Δ 3MST) induced an increase in plasma BUN levels (Fig. 3). The degree

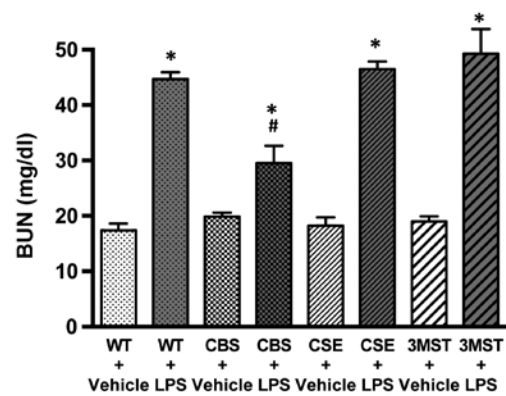


Figure 3. Downregulation of cystathionine- β -synthase (CBS) attenuates the lipopolysaccharide (LPS)-induced increases in blood urea nitrogen (BUN) plasma levels in mice. BUN levels in wild-type (WT), CBS heterozygous (CBS^{+/-}), cystathionine- γ -lyase (CSE) knockout (CSE^{-/-}) and 3-mercaptopyruvate sulfurtransferase (3MST) mutant (Δ 3MST) mice treated with vehicle or LPS (10 mg/kg, 6 h). LPS significantly impaired glomerular function, as evidenced by markedly increased BUN concentration; this increase was reduced in CBS^{+/-} mice. Data are shown as mean \pm standard error of the mean (SEM) of 10 animals; *p<0.05 shows significant increases in response to LPS, compared to the vehicle control; #p<0.05 shows a significant protective effect of the CBS^{+/-} phenotype compared to WT.

of this increase was comparable in the WT, CSE^{-/-} and Δ 3MST mice; however, the CBS^{+/-} mice exhibited a reduced degree of LPS-induced increased plasma BUN levels compared to the WT mice (Fig. 3).

Effect of CBS^{+/-}, CSE^{-/-} and Δ 3MST on LPS-induced MPO and MDA tissue levels. LPS administration induced an increase in lung MPO and MDA levels in all four groups of mice studied (Fig. 4). The degree of the increase in pulmonary MDA post-LPS levels tended to be less in the CSE^{-/-} and CBS^{+/-} mice compared to the WT mice (Fig. 4).

Effect of CBS^{+/-}, CSE^{-/-} and Δ 3MST on LPS-induced plasma cytokine levels. LPS administration induced an increase in the plasma levels of multiple cytokines in all four groups of mice studied (Figs. 5-9). The degree of the increase in TNF α tended to be less in all three groups of mice deficient in various H₂S-producing enzymes (Fig. 5), while plasma IL-5 and GM-CSF levels tended to be higher after LPS challenge in the Δ 3MST mice (Fig. 7). The degree of the increases in IL-10 and IL-12 levels tended to be less in all three groups of mice deficient in various H₂S-producing enzymes (Figs. 8 and 9); plasma IFN γ levels after LPS were lower in CBS^{+/-} mice compared to WT mice (Fig. 9).

Effect of CBS^{+/-}, CSE^{-/-} and Δ 3MST on LPS-induced survival. Survival curves after LPS challenge tended to be shifted to the right in the CSE^{-/-} and CBS^{+/-} mice compared to the WT mice; however, the effect failed to reach statistical significance, while the survival curves of the WT and Δ 3MST mice were superimposable (Fig. 10).

Discussion

The main conclusions of the current study are the following: i) LPS induces a tissue-dependent upregulation of H₂S-pro-

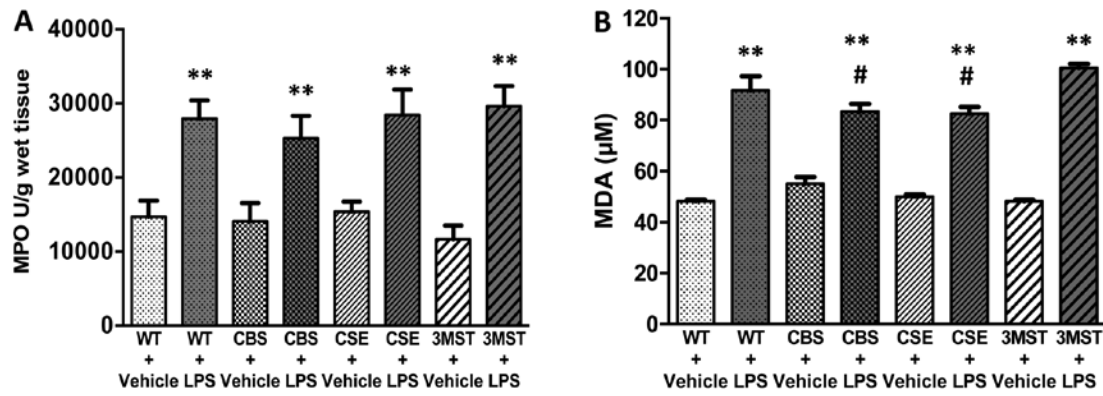


Figure 4. Downregulation of cystathionine- β -synthase (CBS) or cystathionine- γ -lyase (CSE) attenuates the lipopolysaccharide (LPS)-induced increases in lung malondialdehyde (MDA), but not myeloperoxidase (MPO) levels in mice. Lung (A) MPO and (B) MDA levels in wild-type (WT), CBS heterozygous (CBS^{+/-}), CSE knockout (CSE^{-/-}) and 3-mercaptopyruvate sulfurtransferase (3MST) mutant (Δ 3MST) mice treated with vehicle or LPS (10 mg/kg, 6 h). LPS significantly increased MPO and MDA levels; this increase was reduced in CSE^{-/-} and CBS^{+/-} mice. Data are shown as the means \pm standard error of the mean (SEM) of 10 animals; **p<0.01 shows significant increases in response to LPS, compared to the vehicle control; #p<0.05 shows a reduction by the CSE^{-/-} and CBS^{+/-} phenotype compared to WT.

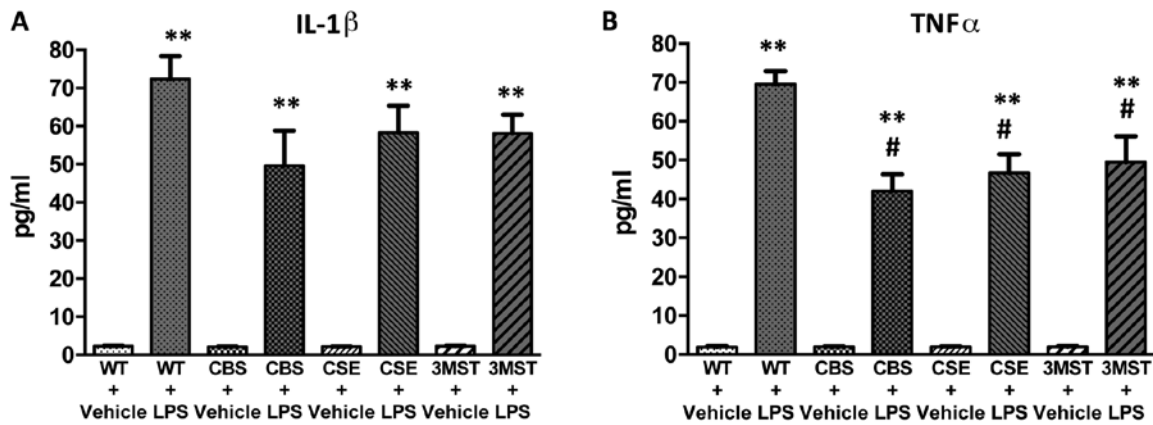


Figure 5. Downregulation of cystathionine- β -synthase (CBS), cystathionine- γ -lyase (CSE) or 3-mercaptopyruvate sulfurtransferase (3MST) attenuates the lipopolysaccharide (LPS)-induced increases in plasma tumor necrosis factor (TNF) α , but not interleukin (IL)-1 β levels in mice. Plasma (A) IL-1 β and (B) TNF α levels in wild-type (WT), CBS heterozygous (CBS^{+/-}), CSE knockout (CSE^{-/-}) and 3MST mutant (Δ 3MST) mice treated with the vehicle or LPS (10 mg/kg, 6 h). LPS significantly increased IL-1 β and TNF α levels; the increase in TNF α was reduced in CBS^{+/-}, CSE^{-/-} and Δ 3MST mice. Data are shown as the means \pm standard error of the mean (SEM) of 10 animals; **p<0.01 shows significant increases in response to LPS, compared to the vehicle control; #p<0.05 shows a significant reduction on TNF α plasma levels in the CSE^{-/-}, CBS^{+/-} and Δ 3MST phenotype compared to WT.

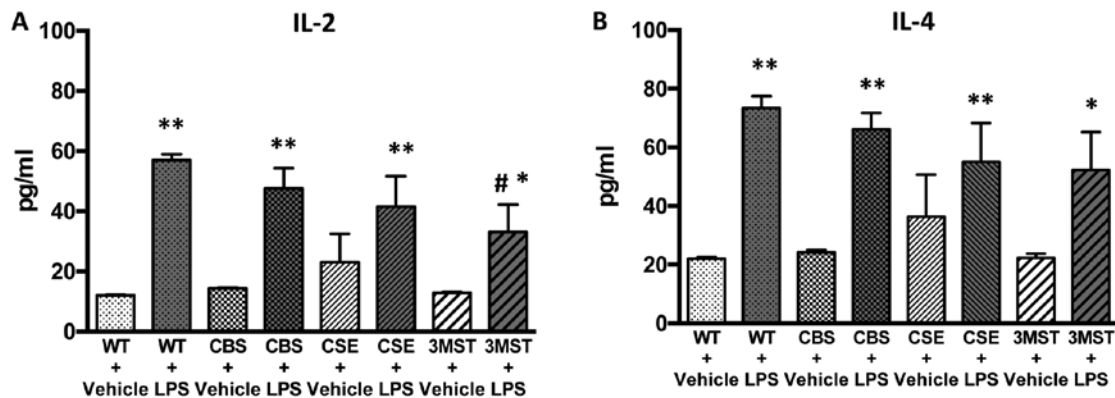


Figure 6. Downregulation of cystathionine- β -synthase (CBS), cystathionine- γ -lyase (CSE) or 3-mercaptopyruvate sulfurtransferase (3MST) does not affect the lipopolysaccharide (LPS)-induced increases in plasma interleukin (IL)-2 or IL-4 levels in mice. Plasma (A) IL-2 and (B) IL-4 levels in wild-type (WT) mice, in CBS heterozygous (CBS^{+/-}), CSE knockout (CSE^{-/-}) and 3MST mutant (Δ 3MST) mice treated with the vehicle or LPS (10 mg/kg, 6 h). LPS significantly increased IL-2 and IL-4 levels; the effect was comparable in all four groups of animals studied. Data are shown as the means \pm standard error of the mean (SEM) of 10 animals; *p<0.05 and **p<0.01 show significant increases in response to LPS, compared to the vehicle control; #p<0.05 shows a significant reduction of IL-2 plasma levels in the Δ 3MST phenotype, compared to WT.

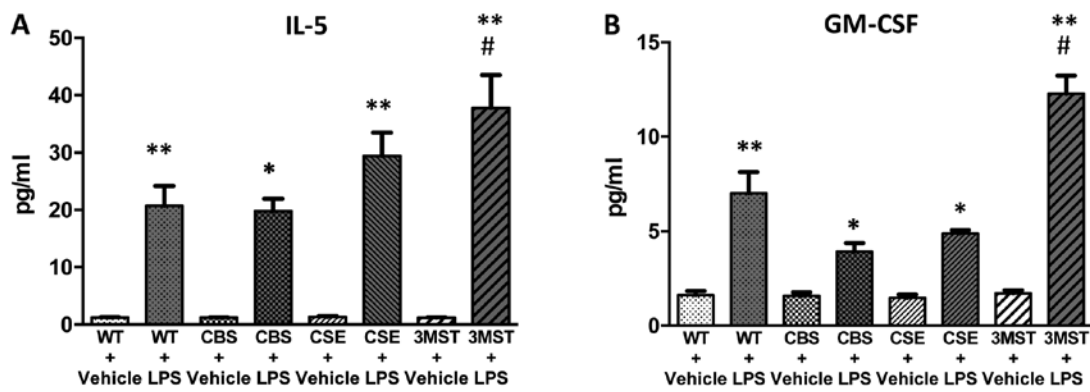


Figure 7. Downregulation of 3-mercaptopyruvate sulfurtransferase (3MST) increases the lipopolysaccharide (LPS)-induced increases in plasma interleukin (IL)-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) levels in mice. Plasma (A) IL-5 and (B) GM-CSF levels in wild-type (WT), cystathionine-β-synthase (CBS) heterozygous (CBS^{+/+}), cystathionine-γ-lyase (CSE) knockout (CSE^{-/-}) and 3MST mutant (Δ3MST) mice treated with the vehicle or LPS (10 mg/kg, 6 h). LPS significantly increased IL-5 and GM-CSF levels; the effect was more pronounced in the Δ3MST mice than in the WT controls. Data are shown as the means ± standard error of the mean (SEM) of 10 animals; *p<0.05 shows significant increases in response to LPS, compared to the vehicle control; #p<0.05 shows significantly higher cytokine responses in the Δ3MST mice compared to the WT controls.

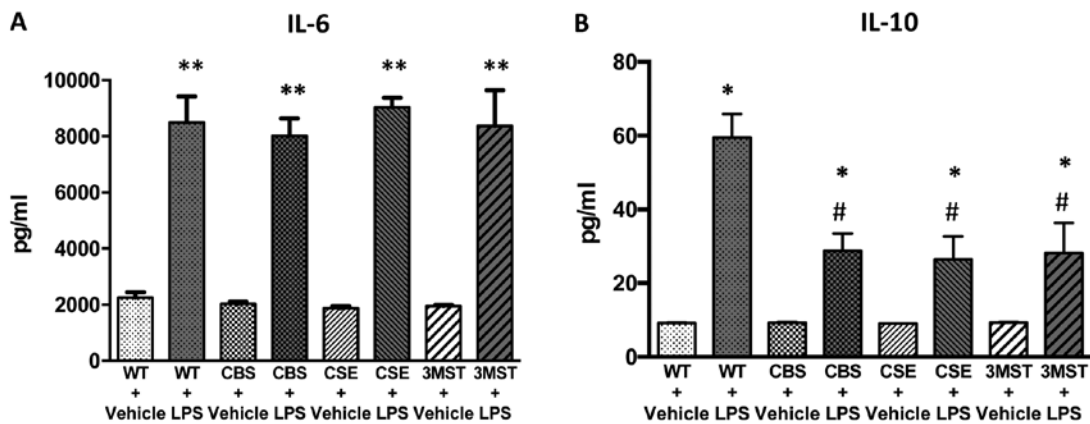


Figure 8. Downregulation of cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) or 3-mercaptopyruvate sulfurtransferase (3MST) attenuates the lipopolysaccharide (LPS)-induced increases in plasma interleukin (IL)-10, but not IL-6 levels in mice. Plasma (A) IL-6 and (B) IL-10 levels in wild-type (WT), CBS heterozygous (CBS^{+/+}), CSE knockout (CSE^{-/-}) and 3MST mutant (Δ3MST) mice treated with the vehicle or LPS (10 mg/kg, 6 h). LPS significantly increased IL-6 and IL-10 levels; the increase in IL-10 was reduced in CBS^{+/+}, CSE^{-/-} and Δ3MST mice. Data are shown as the means ± standard error of the mean (SEM) of 10 animals; *p<0.05 and **p<0.01 show significant increases in response to LPS, compared to the vehicle control; #p<0.05 shows a significant protective effect of the CSE^{-/-}, CBS^{+/+} and Δ3MST phenotype compared to WT.

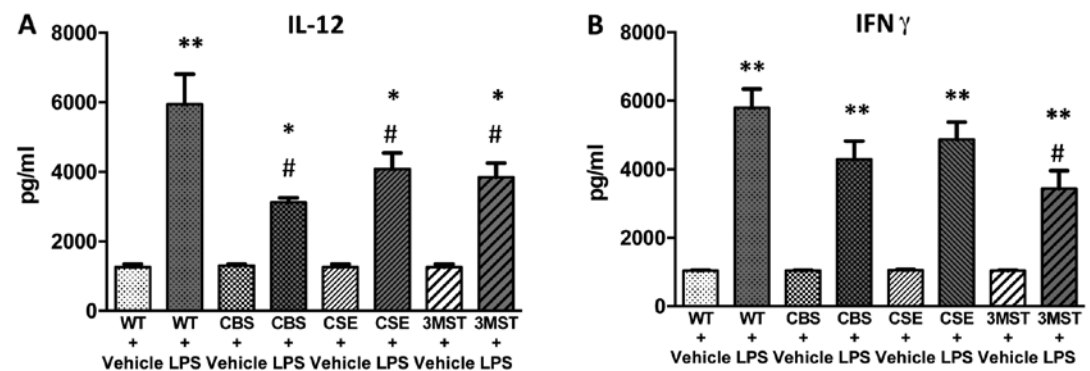


Figure 9. Downregulation of cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) or 3-mercaptopyruvate sulfurtransferase (3MST) attenuates the lipopolysaccharide (LPS)-induced increases in plasma interleukin (IL)-12, while downregulation of CSE reduces plasma interferon (IFN)γ levels in mice. Plasma (A) IL-12 and (B) IFNγ levels in wild-type (WT), CBS heterozygous (CBS^{+/+}), CSE knockout (CSE^{-/-}) and 3MST mutant (Δ3MST) mice treated with the vehicle or LPS (10 mg/kg, 6 h). LPS significantly increased IL-12 and IFNγ levels; the increase in IL-12 was reduced in CBS^{+/+}, CSE^{-/-} and Δ3MST mice while the increase in IFNγ was reduced in the CSE^{-/-} mice. Data are shown as the means ± standard error of the mean (SEM) of 10 animals; *p<0.05 and **p<0.01 show significant increases in response to LPS, compared to the vehicle control; #p<0.05 shows a significant protective effect of the CSE^{-/-}, CBS^{+/+} and Δ3MST phenotype compared to WT on IL-12 levels or the protective effect of CSE^{-/-} compared to WT on IFNγ levels.

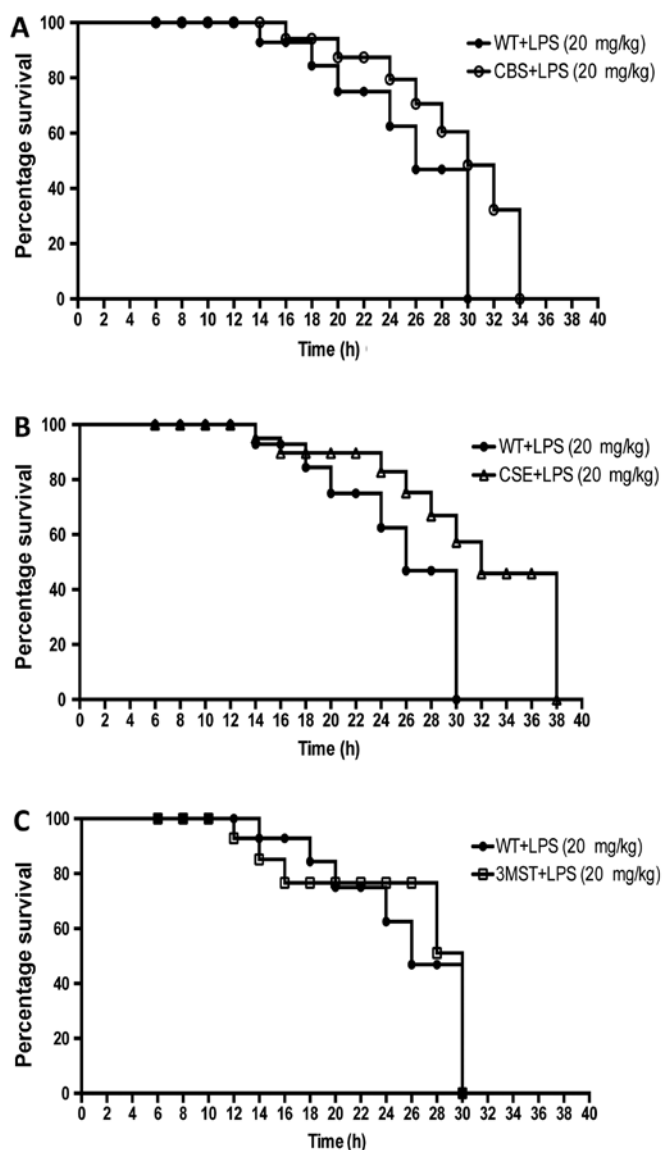


Figure 10. Downregulation of, cystathionine- β -synthase (CBS), cystathionine- γ -lyase (CSE) or 3-mercaptopyruvate sulfurtransferase (3MST) does not affect lipopolysaccharide (LPS)-induced mortality in mice. Comparison of (A) wild-type (WT) and CBS heterozygous (CBS^{+/-}) mice, (B) WT and CSE knockout (CSE^{-/-}) mice, and (C) WT and 3MST mutant (Δ 3MST) mice treated with the vehicle or LPS (20 mg/kg). LPS induced a significant mortality, which was comparable in WT, CSE^{-/-}, CBS^{+/-} and Δ 3MST mice at all time points. Data show survival proportions (%) of n=15 mice in each experimental group.

ducing enzymes in mice (upregulation of CBS in the spleen, upregulation of 3MST in the liver and upregulation of CBS, CSE and 3MST in the lung), with similar (but not identical) patterns observed in genetically modified mice lacking either of the three CBS-producing enzymes; ii) LPS induces the various expected hallmarks of organ injury (elevated BUN levels, elevated tissue levels of MPO and MDA), increased levels of circulating cytokines and mortality over time; iii) the LPS-induced alterations are only slightly or partially affected by the partial or complete absence of any individual H₂S-producing enzymes; with the most pronounced changes being a) a partial attenuation of LPS-induced BUN levels in the CBS^{+/-} mice; b) a partial attenuation of MDA levels in the

of CBS^{+/-} and CSE^{-/-} mice; and c) a partial attenuation of TNF α , IL-10 and IL-12 levels in all three genetically modified animal groups. Finally, iv) there were no statistically significant effects of any of the genetic modifications on LPS-induced mortality. Based on these data, we conclude that a deficiency in any single one of the three major H₂S-producing enzymes only slightly affects the outcome of LPS-induced endotoxemia *in vivo*.

As already mentioned in the 'Introduction', the current body of literature on endotoxemia, endotoxin shock, sepsis, as well as various other forms of critical illness (e.g., burn injury, ARDS and hemorrhagic shock) is fairly controversial with respect to the role of H₂S in the pathogenesis of these diseases; some of the studies have demonstrated that pharmacological H₂S donation is beneficial in some experimental models (11-16), while other studies have concluded that pharmacological inhibitors of H₂S biosynthesis (17-21), or the genetic deletion of CSE (22,23) is beneficial. Moreover, there are also studies demonstrating that H₂S donation can be detrimental (18-20), and there are even studies demonstrating that pharmacological inhibitors of H₂S biosynthesis can be detrimental in certain models of critical illness (31,32). While some of these discrepancies may be attributable to the differences in the experimental models used, some of the explanation is likely to be related to the well-known bell-shaped dose-response character (1-10) of H₂S, where lower concentrations of the mediator exert distinctly different (often opposite) pharmacological effects than higher concentrations; indeed, lower concentrations, and delayed administration of H₂S donors are often found protective, while higher concentrations are often detrimental. It should also be noted that H₂S exerts differential effects on various functions in different cell types and different organs (e.g., vascular functions, pro-inflammatory signaling, redox/oxidant processes, cell death effector pathways, cellular bioenergetic pathways); modulation of some of these effects may be ultimately beneficial for the outcome of critical illness, while modulation of others may be detrimental. Thus, the ultimate outcome parameters may depend on the relative importance of the various pathways affected by H₂S in the particular experimental model studied. The outcome of the experiment may also depend on the changes in endogenous H₂S biosynthesis; for instance, in some (but not all) models of critical illness, H₂S levels can be elevated; these elevated H₂S levels may serve cytoprotective as well as deleterious roles, dependent on the type of critical illness, and perhaps the stage and the severity of the disease as well [reviewed in (6,8,33,34)]. Similarly, H₂S has been demonstrated to affect the production of various pro- and anti-inflammatory cytokines (6,8,11,12-16,19); both stimulatory and inhibitory effects have been reported; the direction of the effect is dependent on the concentration of H₂S used, as well as the experimental model and cell type used, and it has been shown to involve a variety of signaling pathways including NF- κ B, MAP kinases and histone deacetylases (14,19,20,35-39). Thus, there may be multiple mechanistic reasons (in addition to model-dependent differences) why inhibition of H₂S biosynthesis or donation of H₂S can affect the outcome of a complex disease like septic shock in a beneficial or detrimental manner, depending on the constellation of the multitude of the factors and processes discussed above.

As regards the effect of genetic deficiency of H₂S-producing enzymes on the outcome of organ injury, some of the data published in the literature indicate that it can be detrimental:

e.g., CSE deletion in myocardial and hepatic ischemia-reperfusion models exacerbates organ damage (40). Moreover, CSE or CBS deletion in renal injury models increases disease severity (41). However, in two recently published murine models of critical illness, the data indicate that CSE^{-/-} mice are protected against LPS/galactosamine-induced hepatic injury (22) and in a model of cecal ligation and puncture, the specific silencing of CSE in circulating mononuclear cells was found to improve disease outcomes (23). In the current study, while the Δ 3MST mice tended to exhibit similar patterns to the WT mice for most of the key parameters studied (organ damage indices, cytokine profiles, survival), the CBS^{+/-} and CSE^{-/-} mice tended to exhibit slight trends towards protection such as lower BUN levels (CBS^{+/-} mice), in several cases lower cytokine levels (both CBS^{+/-} and CSE^{-/-} mice) and a trend towards delays in LPS-induced mortality. Based on these data, and coupled with the fact that we found that LPS induces an upregulation of various H₂S-producing enzymes in various organs, we conclude that endogenously produced H₂S, in the current model, on the whole, tends to exhibit predominantly a deleterious overall effect. The reduction in some of the plasma cytokines in all three groups of mice deficient in H₂S-producing enzymes corresponds to a mixed pro/anti-inflammatory effect of H₂S biosynthesis inhibition, because both pro-inflammatory (TNF α) and anti-inflammatory (IL-10) mediator production was suppressed. The fact that the effects observed in the current study are often partial (and in many cases do not reach statistical significance) may be attributed to the fact that each of the genetically modified animals used in the current study only has a partial defect in the H₂S production; in some cases the deficiency itself is partial (CBS, 3MST) and even in the animals where the deficiency of the target (CSE) is complete, the remaining H₂S-producing enzymes continue to synthesize H₂S, which may, in some cases, exert compensatory effects. Although there are some differences between the various strains of mice with respect to upregulation of various H₂S-producing enzymes in response to LPS, we do not suggest that the compensation proposed above occurs because the genetically modified mice produce more H₂S via upregulation of various alternative H₂S-producing enzymes; we suggest that this compensation is simply the result of the fact that deletion of either of the three enzymes only reduces tissue H₂S levels to a partial degree.

The purpose of the current study was to determine the effect of each individual H₂S-producing enzyme, separately, on LPS-induced responses. A mouse that is simultaneously deficient in all three enzymes is currently not available - neither in our laboratory nor in other laboratories; there are no published studies in the literature using such an approach. It remains, therefore, to be determined, whether the simultaneous lack of all three H₂S-producing enzymes would change the viability of an animal (under baseline conditions or under various pathophysiological conditions).

We are aware of several limitations of the current study. First of all, we did not use littermate controls for the WT mice. Instead, we used C57/BL6 mice. This is the exactly appropriate control for the CBS^{+/-} mice, as they were obtained from Jackson Laboratory, and have the same background, as well as the 3MST mutant mice which are on the

same background as well. However, the CSE^{-/-} mice were on a mixed background. This is a limitation of the study. Nevertheless, genetic background differences tend to pose more of a problem when there are differences found between the groups of animals compared (as it remains to be determined whether the differences are due to the absence of the enzyme studied, or, perhaps due to background differences). However, in our case, actually, there are no significant differences between the responses of the WT and the CSE^{-/-} mice to LPS. This means that neither the presence/absence of the H₂S-producing enzyme, nor the potential differences due to background make enough difference to culminate in a significant difference in the outcome variables studied. We believe that with the additional discussion and caveats the material presented here continues to contain useful information for the field. Second, only a single time point (6 h post LPS) was studied for the various parameters of renal injury, MPO/MDA and cytokines; since the course of critical illness has several stages, further studies will be necessary to determine whether the effects are different, depending on the timing/stage of the illness. Third, the survival study employed here utilized a severe model, with 100% mortality. Generally, a severe disease model tends to be harder to be affected by therapeutic intervention than a milder model; follow-up studies may employ different models with lower severity. Fourth, the current model only used one particular model of sepsis/shock, the one induced by bacterial endotoxin; other models (e.g., sepsis models induced by live bacteria, or by polymicrobial sepsis, e.g., the one induced by cecal ligation and puncture) may yield a more complete picture. Fifth, in the current study some of the H₂S-producing enzymes we sought to study were only partially downregulated due to technical/practical issues - e.g., CBS^{-/-} mice have a very high mortality rate early on after birth, and the large majority of the animals do not live until young-adult age to be suitable for the LPS model utilized here (42); the mutation in 3MST gene only produced a partial and tissue-dependent reduction in 3MST levels in the strain of 3MST mice we have had access to. Naturally, since the strain used in the current study does not have a downregulation of 3MST in the liver or the kidney, we did not expect that WT vs. 3MST mutant mice will respond differently to LPS-induced liver or kidney dysfunction; and, indeed, they did not. There are other models of CBS deficiency in mice, e.g., a model where CBS is completely absent, and the mouse is engineered to contain a deficient human form of CBS (42), that may be better suitable for future studies; likewise, a group in Japan has created a full 3MST knockout line (43); these genetically modified animals may be useful in future studies. Sixth, in the current study, we did not measure circulating H₂S levels, only the tissue expression of various H₂S-producing enzymes. We do not feel that measurement of circulating H₂S levels would be particularly valuable in the context of the current study, given the fact that multiple organ-specific changes were demonstrated in the expression of the various H₂S-producing enzymes after LPS. In addition, there are many prior studies indicating that the net level of circulating H₂S is not predictable for the outcome of critical illness, since both H₂S donors and H₂S biosynthesis inhibitors have demonstrated beneficial effects in various models (6,8,11-23,31-33); it has

been suggested that the timing of the donation or inhibition as well as possible regional (cell- and organ-specific differences likely play a role). In the current study, we only used mice with global deficiency of the target enzymes; given the multiple, cell-, tissue- and organ-specific biological roles of H₂S, future studies with cell-type selective deletion of various H₂S-producing enzymes may also be highly instrumental to unveil the complex roles of H₂S and H₂S-producing enzymes in various forms of critical illness.

Acknowledgements

This study was supported by the National Institutes of Health (R01GM107846) to C.S.

References

- Fiorucci S, Distrutti E, Cirino G and Wallace JL: The emerging roles of hydrogen sulfide in the gastrointestinal tract and liver. *Gastroenterology* 131: 259-271, 2006.
- Szabó C: Hydrogen sulphide and its therapeutic potential. *Nat Rev Drug Discov* 6: 917-935, 2007.
- Wang R: Physiological implications of hydrogen sulfide: A whiff exploration that blossomed. *Physiol Rev* 92: 791-896, 2012.
- Predmore BL, Lefer DJ and Gojon G: Hydrogen sulfide in biochemistry and medicine. *Antioxid Redox Signal* 17: 119-140, 2012.
- Vandiver M and Snyder SH: Hydrogen sulfide: A gasotransmitter of clinical relevance. *J Mol Med (Berl)* 90: 255-263, 2012.
- Coletta C and Szabo C: Potential role of hydrogen sulfide in the pathogenesis of vascular dysfunction in septic shock. *Curr Vasc Pharmacol* 11: 208-221, 2013.
- Szabo C, Ransy C, Módis K, Andriamihaja M, Murghes B, Coletta C, Olah G, Yanagi K and Bouillaud F: Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part I. Biochemical and physiological mechanisms. *Br J Pharmacol* 171: 2099-2122, 2014.
- McCook O, Radermacher P, Volani C, Asfar P, Ignatius A, Kemmler J, Möller P, Szabó C, Whiteman M, Wood ME, *et al*: H₂S during circulatory shock: Some unresolved questions. *Nitric Oxide* 41: 48-61, 2014.
- Kimura H: Signaling molecules: Hydrogen sulfide and polysulfide. *Antioxid Redox Signal* 22: 362-376, 2015.
- Huang CW and Moore PK: 2S synthesizing enzymes: Biochemistry and molecular aspects. *Handb Exp Pharmacol* 230: 3-25, 2015.
- Li L, Salto-Tellez M, Tan CH, Whiteman M and Moore PK: GYY4137, a novel hydrogen sulfide-releasing molecule, protects against endotoxemic shock in the rat. *Free Radic Biol Med* 47: 103-113, 2009.
- Tokuda K, Kida K, Marutani E, Crimi E, Bougaki M, Khatri A, Kimura H and Ichinose F: Inhaled hydrogen sulfide prevents endotoxin-induced systemic inflammation and improves survival by altering sulfide metabolism in mice. *Antioxid Redox Signal* 17: 11-21, 2012.
- Aslami H, Beurskens CJ, de Beer FM, Kuipers MT, Roelofs JJ, Hegeman MA, Van der Sluijs KF, Schultz MJ and Juffermans NP: A short course of infusion of a hydrogen sulfide-donor attenuates endotoxemia induced organ injury via stimulation of anti-inflammatory pathways, with no additional protection from prolonged infusion. *Cytokine* 61: 614-621, 2013.
- Chen X, Xu W, Wang Y, Luo H, Quan S, Zhou J, Yang N, Zhang T, Wu L, Liu J, *et al*: Hydrogen sulfide reduces kidney injury due to urinary-derived sepsis by inhibiting NF- κ B expression, decreasing TNF- α levels and increasing IL-10 levels. *Exp Ther Med* 8: 464-470, 2014.
- Ferlito M, Wang Q, Fulton WB, Colombani PM, Marchionni L, Fox-Talbot K, Paolucci N and Steenbergen C: Hydrogen sulfide [corrected] increases survival during sepsis: Protective effect of CHOP inhibition. *J Immunol* 192: 1806-1814, 2014.
- Ahmad A, Druzhyna N and Szabo C: Delayed treatment with sodium hydrosulfide improves regional blood flow and alleviates cecal ligation and puncture (CLP)-induced septic shock. *Shock* 46: 183-193, 2016.
- Collin M, Anuar FB, Murch O, Bhatia M, Moore PK and Thiernemann C: Inhibition of endogenous hydrogen sulfide formation reduces the organ injury caused by endotoxemia. *Br J Pharmacol* 146: 498-505, 2005.
- Li L, Bhatia M, Zhu YZ, Zhu YC, Ramnath RD, Wang ZJ, Anuar FB, Whiteman M, Salto-Tellez M and Moore PK: Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. *FASEB J* 19: 1196-1198, 2005.
- Zhang H, Zhi L, Moochhala S, Moore PK and Bhatia M: Hydrogen sulfide acts as an inflammatory mediator in cecal ligation and puncture-induced sepsis in mice by upregulating the production of cytokines and chemokines via NF-kappaB. *Am J Physiol Lung Cell Mol Physiol* 292: L960-L971, 2007.
- Zhang H, Moochhala SM and Bhatia M: Endogenous hydrogen sulfide regulates inflammatory response by activating the ERK pathway in polymicrobial sepsis. *J Immunol* 181: 4320-4331, 2008.
- Yan Y, Chen C, Zhou H, Gao H, Chen L, Chen L, Gao L, Zhao R and Sun Y: Endogenous hydrogen sulfide formation mediates the liver damage in endotoxemic rats. *Res Vet Sci* 94: 590-595, 2013.
- Shirozu K, Tokuda K, Marutani E, Lefer D, Wang R and Ichinose F: Cystathionine γ -lyase deficiency protects mice from galactosamine/lipopolysaccharide-induced acute liver failure. *Antioxid Redox Signal* 20: 204-216, 2014.
- Badie E, Chambers ST, Gaddam RR and Bhatia M: Cystathionine- γ -lyase gene silencing with siRNA in monocytes/macrophages attenuates inflammation in cecal ligation and puncture-induced sepsis in the mouse. *J Biosci* 41: 87-95, 2016.
- Watanabe M, Osada J, Aratani Y, Kluckman K, Reddick R, Malinow MR and Maeda N: Mice deficient in cystathionine beta-synthase: Animal models for mild and severe homocyst(e) inemia. *Proc Natl Acad Sci USA* 92: 1585-1589, 1995.
- Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, *et al*: H₂S as a physiological vasorelaxant: Hypertension in mice with deletion of cystathionine gamma-lyase. *Science* 322: 587-590, 2008.
- Gero D, Ahmad A, Brunyanszki A, Olah G, Szczesny B and Szabo C: 3-Mercaptopyruvate sulfurtransferase deficient mice show accelerated glucose uptake and a dysregulated metabolic profile. *Nitric Oxide* 47: S35-S36, 2015.
- Jagtap P, Soriano FG, Virág L, Liaudet L, Mabley J, Szabó E, Haskó G, Marton A, Lorigados CB, Gallyas F Jr, *et al*: Novel phenanthridinone inhibitors of poly (adenosine 5'-diphosphate-ribose) synthetase: Potent cytoprotective and antishock agents. *Crit Care Med* 30: 1071-1082, 2002.
- Namas RA, Bartels J, Hoffman R, Barclay D, Billiar TR, Zamora R and Vodovotz Y: Combined in silico, in vivo, and in vitro studies shed insights into the acute inflammatory response in middle-aged mice. *PLoS One* 8: e67419, 2013.
- Bhargava R, Altmann CJ, Andres-Hernando A, Webb RG, Okamura K, Yang Y, Falk S, Schmidt EP and Faubel S: Acute lung injury and acute kidney injury are established by four hours in experimental sepsis and are improved with pre, but not post, sepsis administration of TNF- α antibodies. *PLoS One* 8: e79037, 2013.
- Everhardt Queen A, Moerdyk-Schauwecker M, McKee LM, Leamy LJ and Huet YM: Differential expression of inflammatory cytokines and stress genes in male and female mice in response to a lipopolysaccharide challenge. *PLoS One* 11: e0152289, 2016.
- Zhang J, Sio SW, Moochhala S and Bhatia M: Role of hydrogen sulfide in severe burn injury-induced inflammation in mice. *Mol Med* 16: 417-424, 2010.
- Bekpinar S, Unlucerci Y, Uysal M and Gurdol F: Propargylglycine aggravates liver damage in LPS-treated rats: Possible relation of nitrosative stress with the inhibition of H₂S formation. *Pharmacol Rep* 66: 897-901, 2014.
- Módis K, Bos EM, Calzia E, van Goor H, Coletta C, Papapetropoulos A, Hellmich MR, Radermacher P, Bouillaud F and Szabo C: Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part II. Pathophysiological and therapeutic aspects. *Br J Pharmacol* 171: 2123-2146, 2014.
- Brunyanszki A, Erdelyi K, Szczesny B, Olah G, Salomao R, Herndon DN and Szabo C: Upregulation and mitochondrial sequestration of hemoglobins occurs in circulating leukocytes during critical illness, conferring a cytoprotective phenotype. *Mol Med (In press)*.
- Hu LF, Wong PT, Moore PK and Bian JS: Hydrogen sulfide attenuates lipopolysaccharide-induced inflammation by inhibition of p38 mitogen-activated protein kinase in microglia. *J Neurochem* 100: 1121-1128, 2007.

36. Chi XP, Ouyang XY and Wang YX: Hydrogen sulfide synergistically upregulates *Porphyromonas gingivalis* lipopolysaccharide-induced expression of IL-6 and IL-8 via NF- κ B signalling in periodontal fibroblasts. *Arch Oral Biol* 59: 954-961, 2014.
37. Rios EC, Szczesny B, Soriano FG, Olah G and Szabo C: Hydrogen sulfide attenuates cytokine production through the modulation of chromatin remodeling. *Int J Mol Med* 35: 1741-1746, 2015.
38. Lee HH, Han MH, Hwang HJ, Kim GY, Moon SK, Hyun JW, Kim WJ and Choi YH: Diallyl trisulfide exerts anti-inflammatory effects in lipopolysaccharide-stimulated RAW 264.7 macrophages by suppressing the Toll-like receptor 4/nuclear factor- κ B pathway. *Int J Mol Med* 35: 487-495, 2015.
39. Yang H, Wang H, Ju Z, Ragab AA, Lundbäck P, Long W, Valdes-Ferrer SI, He M, Pribis JP, Li J, *et al*: MD-2 is required for disulfide HMGB1-dependent TLR4 signaling. *J Exp Med* 212: 5-14, 2015.
40. King AL, Polhemus DJ, Bhushan S, Otsuka H, Kondo K, Nicholson CK, Bradley JM, Islam KN, Calvert JW, Tao YX, *et al*: Hydrogen sulfide cytoprotective signaling is endothelial nitric oxide synthase-nitric oxide dependent. *Proc Natl Acad Sci USA* 111: 3182-3187, 2014.
41. Wang P, Isaak CK, Siow YL and O K: Downregulation of cystathionine β -synthase and cystathionine γ -lyase expression stimulates inflammation in kidney ischemia-reperfusion injury. *Physiol Rep* 2: e12251, 2014.
42. Wang L, Chen X, Tang B, Hua X, Klein-Szanto A and Kruger WD: Expression of mutant human cystathionine beta-synthase rescues neonatal lethality but not homocystinuria in a mouse model. *Hum Mol Genet* 14: 2201-2208, 2005.
43. Nagahara N, Nagano M, Ito T, Shimamura K, Akimoto T and Suzuki H: Antioxidant enzyme, 3-mercaptopyruvate sulfurtransferase-knockout mice exhibit increased anxiety-like behaviors: A model for human mercaptolactate-cysteine disulfiduria. *Sci Rep* 3: 1986, 2013.