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

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Abstract. Hydrogen sulfide (H₂S) 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 H₂S-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 H₂S-producing enzymes (CBS, CSE and 3MST) in the spleen, lung, liver and kidney. LPS induced a tissue-dependent upregulation of some of the H₂S-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 Δ3MST 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 Δ3MST 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)-1β, IL-6, IL-10, IL-12 and interferon (IFN)γ] in plasma; TNFα, IL-10 and IL-12 levels tended to be lower in all three groups of animals expressing lower levels of H₂S-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 (H₂S)-producing enzymes have been identified: cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3MST) (1-10). H₂S is known to regulate a multitude of physiological and pathophysiological functions in the vascular, immune and nervous system (1-10).

The role of H₂S 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 H₂S donation in various models of circulatory shock (11-16), while others have reported that the pharmacological inhibition of H₂S production (17-21) or the genetic deficiency of H₂S-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 H₂S-producing enzymes, CBS, CSE or 3MST. We compared the effect of bacterial LPS in WT, CBS heterozygous (CBS⁺), CSE knockout (CSE⁻) or 3MST mutant (Δ3MST) 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

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Abbreviations: BCA, bicinchoninic acid; BUN, blood urea nitrogen; CBS, cystathionine-β-synthase; CSE, cystathionine-γ-lyase; H₂S, 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 [\(\Delta 3\text{MST}\); 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) \(\Delta 3\text{MST}\) mice + vehicle (n=10); and viii) \(\Delta 3\text{MST}\) 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 µg/µl) 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. Following electrophoretic separation, the proteins were electrophoresed on 8-12% polyacrylamide gels. Electrophoresed gels were stained with Coomassie brilliant blue. The band intensity of the original blots was quantified using the ImageQuant software (Thermo Fisher Scientific). The band intensity of the original blots was quantified using the ImageQuant software (Thermo Fisher Scientific). The band intensity of the original blots was quantified using the ImageQuant software (Thermo Fisher Scientific). The band intensity of the original blots was quantified using the ImageQuant software (Thermo Fisher Scientific). The band intensity of the original blots was quantified using the ImageQuant software (Thermo Fisher Scientific). The band intensity of the original blots was quantified using the ImageQuant software (Thermo Fisher Scientific). The band intensity of the original blots was quantified using the ImageQuant software (Thermo Fisher Scientific).

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 fluorometric 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 aliquoted, 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)-1β, 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 µm) or 80 magnetic microspheres (6.45 µm), 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-phycocerythrin 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 (\(\Delta 3\text{MST}\)) 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.
Statistical analysis. All values described in the text and figures are expressed as the means ± 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 H$_2$S-producing enzymes in response to LPS. First, the effect of LPS on the expression of the three H$_2$S-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 Δ3MST 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 Δ3MST 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 Δ3MST 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 figure 1. Baseline tissue expression of cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptoppyruvate 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 (Δ3MST) 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 Δ3MST mice show reduced 3MST expression in the spleen and lung. Data are shown as the means ± standard error of the mean (SEM) of n=5 determinations.
Table 1. Expression profiles of cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptoppyruvate sulfurtransferase (3MST) at 6 h after the lipopolysaccharide (LPS) (10 mg/kg) injection in wild-type (WT), CBS+/−, CSE+/− and Δ3MST mice.

|                    | WT (%) | CBS+/− (%) | CSE+/− (%) | Δ3MST (%) |
|--------------------|--------|------------|------------|-----------|
| CBS expression     |        |            |            |           |
| Spleen             | 227±29a| 168±15a    | 111±6      | 107±15    |
| Lung               | 134±12a| 115±6      | 109±17     | 120±10    |
| Liver              | 112±12a| 19±6       | 149±11a    | 107±13    |
| Kidney             | 112±14a| 19±5       | 161±15a    | 123±9a    |
| CSE expression     |        |            |            |           |
| Spleen             | 116±14a| 125±15a    | 0          | 91±9      |
| Lung               | 134±19a| 92±16      | 0          | 120±6a    |
| Liver              | 92±5   | 101±6      | 0          | 103±22    |
| Kidney             | 119±1  | 106±1,6    | 0          | 103±12    |
| 3MST expression    |        |            |            |           |
| Spleen             | 116±8  | 125±10a    | 125±11a    | 1±1       |
| Lung               | 177±25a| 159±20a    | 241±14a    | 9±1       |
| Liver              | 139±9a | 129±12a    | 111±11     | 107±13    |
| Kidney             | 110±13 | 114±8      | 9±1        | 115±13    |

*p<0.05 shows significant change compared to baseline control in wild-type mice (which is considered as 100%). Data are shown as the means ± 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-mercaptoppyruvate 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 ± 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 1).

Effect of CBS+/−, CSE+/− and Δ3MST on LPS-induced blood urea nitrogen (BUN) plasma levels. LPS administration to all four groups of mice studied (WT, CBS+/−, CSE+/− and Δ3MST) induced an increase in plasma BUN levels (Fig. 3). The degree of this increase was comparable in the WT, CSE+/− and Δ3MST 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 Δ3MST 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 Δ3MST 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 TNFα 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 Δ3MST 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 IFNγ levels after LPS were lower in CBS+/− mice compared to WT mice (Fig. 9).

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-mercaptoppyruvate sulfurtransferase (3MST) mutant (Δ3MST) 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 ± 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.

Effect of CBS+/−, CSE+/− and Δ3MST 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 Δ3MST 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-mercaptopurine sulfurtransferase (3MST) mutant (Δ3MST) 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 ± 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-mercaptopurine sulfurtransferase (3MST) attenuates the lipopolysaccharide (LPS)-induced increases in plasma tumor necrosis factor (TNF)α, but not interleukin (IL)-1β levels in mice. Plasma (A) IL-1β and (B) TNFα 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-1β and TNFα levels; the increase in TNFα was reduced in CBS+/-, CSE-/- and Δ3MST mice. Data are shown as the means ± standard error of the mean (SEM) of 10 animals; *p<0.01 shows significant increases in response to LPS, compared to the vehicle control; #p<0.05 shows a significant reduction on TNFα plasma levels in the CSE-/-, CBS+/- and Δ3MST phenotype compared to WT.

Figure 6. Downregulation of cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) or 3-mercaptopurine 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 (Δ3MST) 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 ± 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 Δ3MST 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 (IFNγ) 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.
of CBS<sup>−/−</sup> and CSE<sup>−/−</sup> mice; and c) a partial attenuation of TNFα, 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 H<sub>2</sub>S-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 H<sub>2</sub>S in the pathogenesis of these diseases; some of the studies have demonstrated that pharmacological H<sub>2</sub>S donation is beneficial in some experimental models (11-16), while other studies have concluded that pharmacological inhibitors of H<sub>2</sub>S biosynthesis (17-21), or the genetic deletion of CSE (22,23) is beneficial. Moreover, there are also studies demonstrating that H<sub>2</sub>S donation can be detrimental (18-20), and there are even studies demonstrating that pharmacological inhibitors of H<sub>2</sub>S 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 H<sub>2</sub>S, where lower concentrations of the mediator exert distinctly different (often opposite) pharmacological effects than higher concentrations; indeed, lower concentrations, and delayed administration of H<sub>2</sub>S donors are often found protective, while higher concentrations are often detrimental. It should also be noted that H<sub>2</sub>S 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 H<sub>2</sub>S in the particular experimental model studied. The outcome of the experiment may also depend on the changes in endogenous H<sub>2</sub>S biosynthesis; for instance, in some (but not all) models of critical illness, H<sub>2</sub>S levels can be elevated; these elevated H<sub>2</sub>S 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, H<sub>2</sub>S 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 H<sub>2</sub>S 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-κB, 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 H<sub>2</sub>S biosynthesis or donation of H<sub>2</sub>S 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 H<sub>2</sub>S-producing enzymes on the outcome of organ injury, some of the data published in the literature indicate that it can be detrimental:

Figure 10. Downregulation of cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) or 3-mercaptoppyruvate sulfurtransferase (3MST) does not affect lipopolysaccharide (LPS)-induced mortality in mice. Comparison of (A) wild-type (WT) and CBS heterozygous (CBS<sup>+</sup>) mice, (B) WT and CSE knockout (CSE<sup>−</sup>) mice, and (C) WT and 3MST mutant (Δ3MST) mice treated with the vehicle or LPS (20 mg/kg). LPS induced a significant mortality, which was comparable in WT, CSE<sup>−</sup>, CBS<sup>+</sup> and Δ3MST mice at all time points. Data show survival proportions (%) of n=15 mice in each experimental group.
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 Δ3MST 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 (TNFα) 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 $H_2S$, future studies with cell-type selective deletion of various $H_2S$-producing enzymes may also be highly instrumental to unveil the complex roles of $H_2S$ and $H_2S$-producing enzymes in various forms of critical illness.

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