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Endogenous Hydrogen Sulfide Regulates Inflammatory Response by Activating the ERK Pathway in Polymicrobial Sepsis[1]

Huili Zhang,[2]* Shabbir M. Moochhala,† and Madhav Bhatia[3]*

Hydrogen sulfide (H_2S) up-regulates inflammatory response in several inflammatory diseases. However, to date, little is known about the molecular mechanism by which H_2S provokes the inflammatory response in sepsis. Thus, the aim of this study was to investigate the signaling pathway underlying the proinflammatory role of H_2S in cecal ligation and puncture (CLP)-induced sepsis. Male Swiss mice were subjected to CLP and treated with DL-propargylglycine (PAG; 50 mg/kg i.p., an inhibitor of H_2S formation), NaHS (10 mg/kg, i.p., an H_2S donor), or saline. PAG was administered 1 h before CLP, whereas NaHS was given at the time of CLP. CLP-induced sepsis resulted in a time-dependent increase in the synthesis of endogenous H_2S. Maximum phosphorylation of ERK1/2 and degradation of IκBα in lung and liver were observed 4 h after CLP. Inhibition of H_2S formation by PAG significantly reduced the phosphorylation of ERK1/2 in lung and liver 4 h after CLP, coupled with decreased degradation of IκBα and activation of NF-κB. In contrast, injection of NaHS significantly enhanced the activation of ERK1/2 in lung and liver, therefore leading to a further rise in tissue NF-κB activity. As a result, pretreatment with PAG significantly reduced the production of cytokines and chemokines in sepsis, whereas exogenous H_2S greatly increased it. In addition, pretreatment with PD98059, an inhibitor of ERK kinase (MEK-1), significantly prevented NaHS from aggravating systemic inflammation in sepsis. In conclusion, the present study shows for the first time that H_2S may regulate systemic inflammatory response in sepsis via ERK pathway. ***The Journal of Immunology,*** **2008, 181: 4320–4331.**

Hydrogen sulfide (H_2S)[4] was traditionally considered to be a toxic gas with the smell of rotten eggs. However, it is generated endogenously during cysteine metabolism in a reaction catalyzed by two pyridoxal phosphate-dependent enzymes, cystathionine β-synthase (CBS; EC4.2.1.22), and/or cystathionine γ-lyase (CSE; EC4.4.1.1) (1–3). Although CBS and CSE are widely distributed in tissues, CBS is the main H_2S-forming enzyme in CNS, whereas CSE is the major H_2S-producing enzyme in the cardiovascular system. Recently, it has become clear that H_2S fulfills a wide range of physiological functions and plays an important role in several pathological conditions. For example, H_2S opens K^+_{ATP} channels in vascular smooth muscle cells, gastrointestinal smooth muscle cells, cardiomyocytes, neurons, and pancreatic β cells, therefore regulating vascular tone, intestinal contractility, myocardial contractility, neurotransmission, and insulin secretion (1–3). In nervous system, H_2S promotes hippocampal long-term potentiation by enhancing the sensitivity of *N*-methyl-D-aspartic acid receptors to glutamate (1–3). Furthermore, H_2S has recently been shown to play an important role in inflammatory response (3).

Sepsis is a common and serious medical condition caused by a severe systemic infection leading to a systemic inflammatory response, which frequently occurs after hemorrhage, trauma, burn, or abdominal surgery (4, 5). Sepsis and its sequelae are leading causes of morbidity and mortality in medical and surgical intensive care units (4, 5). Recent studies have indicated that H_2S may contribute to inflammatory response and regulate the severity of cecal ligation and puncture (CLP)-induced sepsis (6, 7) or LPS-induced endotoxemia (8, 9). In these studies, tissue CSE activity and expression of CSE mRNA were up-regulated, leading to overproduction of endogenous H_2S, whereas DL-propargylglycine (PAG), a CSE inhibitor, exhibited an obvious anti-inflammatory activity and protected animals against sepsis and associated multiple organ injury. Similar observations were also obtained in other inflammatory conditions, such as hindpaw edema (10) and acute pancreatitis (11). Recently, our group has demonstrated that H_2S may up-regulate the production of proinflammatory mediators in sepsis by inducing the translocation and activation of NF-κB (7). Although this study suggests the potential association between NF-κB and H_2S in sepsis, the underlying signaling pathway remains unknown.

In sepsis, LPS, cytokines, and chemokines lead to the activation of various MAPK signaling networks, thereby contributing to the induction of NF-κB and transcription of proinflammatory factors (12, 13). Notably, activation of ERK has been shown to be an essential temporal regulator of NF-κB activity and NF-κB-induced gene expression in response to IL-1 (14). Removal of ERK1/2 by specific inhibitors reduced the activation of NF-κB, suppressed the transcription of NF-κB-dependent genes, and protected against LPS-induced endotoxemia (14–16). In light of these findings, the

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[4] Abbreviations used in this paper: H_2S, hydrogen sulfide; ALT, alanine aminotransferase; CBS, cystathionine β-synthase; CLP, cecal ligation and puncture; CSE, cystathionine γ-lyase; HPRT, hypoxanthine-guanine phosphoribosyltransferase; MPO, myeloperoxidase; PAG, DL-propargylglycine; PKA, protein kinase A.

present study was aimed to elucidate whether H_2S regulates inflammatory response via the ERK-NF-κB pathway in sepsis. Sepsis was induced by CLP, which closely resembles the clinical observations of vascular reactivity and inflammation during polymicrobial peritonitis, bacteremia, and systemic sepsis, both qualitatively and quantitatively (17).

Materials and Methods

Induction of sepsis

All experiments were approved by the animal ethics committee of National University of Singapore and conducted in accordance with the established "Guiding Principles for Animal Research." The previously described model of CLP-induced sepsis was used with minor modifications (18). Male Swiss albino mice (25–30 g) were lightly anesthetized with the mixture of ketamine and medetomidine (0.75 ml of ketamine (100 mg/ml) and 1 ml of medetomidine (1 mg/ml) dissolved in 8.25 ml of distilled water) (7.5 ml/kg) under aseptic conditions. After shaving the abdominal fur and applying a topical disinfectant, a small midline incision was made through the skin and peritoneum of the abdomen to expose the cecum. The cecal appendage was ligated 3–5 mm below the ileocecal valve with Silkam 4/0 thread without occluding the bowel passage, and then perforated at two locations with a 22-gauge needle distal to the point of ligation. After this, a small amount of stool was squeezed out through both the holes. Finally, the bowel was repositioned, and the abdomen was stitched up with sterile Permilene 5/0 thread. Animals with sham operation underwent the same procedure without CLP. PAG (50 mg/kg, i.p.; Sigma-Aldrich), an irreversible inhibitor of CSE, or saline was administered 1 h before CLP or sham operation (8, 10). PD98058, a selective inhibitor of MEK-1 (10 mg/kg, i.p.; Calbiochem), or vehicle (DMSO/0.9%NaCl (1:50), 5 ml/kg, i.p.) was given to mice 1 h before CLP (19, 20). NaHS (10 mg/kg, i.p.; Sigma-Aldrich), an H_2S donor, or saline was given to mice at the time of CLP or sham operation. Four hours after the operation, animals were sacrificed by an i.p. injection of a lethal dose of pentobarbitone (90 mg/kg). Samples of lung and liver were collected and stored at −80°C for subsequent measurement.

Time course study of plasma H_2S level and liver CSE activity after CLP challenge

Mice were divided into two groups and subjected to either CLP or sham operation. Mice were sacrificed 0.5, 2, 4, 8, 16, and 24 h after CLP or sham operation, and the samples of plasma, lung, and liver were collected and stored at −80°C for subsequent measurement.

Measurement of plasma H_2S

Aliquots (120 μl) of plasma were mixed with distilled water (100 μl), trichloroacetic acid (10% w/v, 120 μl), zinc acetate (1% w/v, 60 μl), *N*,*N*-dimethyl-*p*-phenylenediamine sulfate (20 μM; 40 μl) in 7.2 M HCl, and $FeCl_3$ (30 μM; 40 μl) in 1.2 M HCl in 96-well plates. The absorbance of the resulting solution was measured 10 min thereafter at 670 nm (21–23). All samples were assayed in duplicate, and H_2S was calculated against a calibration curve of NaHS (3.125–100 μM). Results show plasma H_2S concentration in μM.

Assay of liver H_2S-synthesizing activity

H_2S-synthesizing activity in liver homogenates was measured essentially as described elsewhere (8). Briefly, liver tissue was homogenized in 100 mM ice-cold potassium phosphate buffer (pH 7.4). The assay mixture contained 100 mM potassium phosphate buffer (pH 7.4), L-cysteine (20 μl, 20 mM), pyridoxal 5′-phosphate (20 μl, 2 mM), saline (30 μl), and 4.5% w/v tissue homogenate (430 μl). The reaction was performed in tightly sealed microcentrifuge tubes and initiated by transferring the tubes from ice to a water bath at 37°C. After incubation for 30 min, 250 μl of zinc acetate (1% w/v) was added to trap-evolved H_2S, followed by 250 μl of trichloroacetic acid (10% v/v) to denature the protein and stop the reaction. Subsequently, *N*,*N*-dimethyl-*p*-phenylenediamine sulfate (20 μM; 133 μl) in 7.2 M HCl was added, immediately followed by $FeCl_3$ (30 μM; 133 μl) in 1.2 M HCl. The absorbance of the resulting solution at 670 nm was measured by spectrophotometry (Tecan Systems) in a 96-well microplate reader (21–23). The H_2S concentration was calculated against a calibration curve of NaHS. Results were then corrected for the DNA content of the tissue sample (24) and expressed as nmoles of H_2S formed/mg DNA.

Myeloperoxidase (MPO) estimation

Tissue samples were thawed, homogenized in 20 mM phosphate buffer (pH 7.4), and centrifuged (13,000 × *g*, 10 min, 4°C), and the resulting pellets were resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% w/v hexadecyltrimethylammonium bromide (Sigma-Aldrich). The suspension was subjected to four cycles of freezing and thawing, and further disrupted by sonication (40 s). The samples were then centrifuged (13,000 × *g*, 5 min, 4°C), and the supernatants were used for the MPO assay. The reaction mixture consisted of the supernatant (50 μl), 1.6 mM tetramethylbenzidine (Sigma-Aldrich), 80 mM sodium phosphate buffer (pH 5.4), and 0.3 mM hydrogen peroxide (reagent volume: 50 μl). This mixture was incubated at 37°C for 110 s. The reaction was terminated with 50 μl of 0.18 M H_2SO_4, and the absorbance was measured at 405 nm. This absorbance was then corrected for the DNA content of the tissue sample (24), and the results were expressed as enzyme activity.

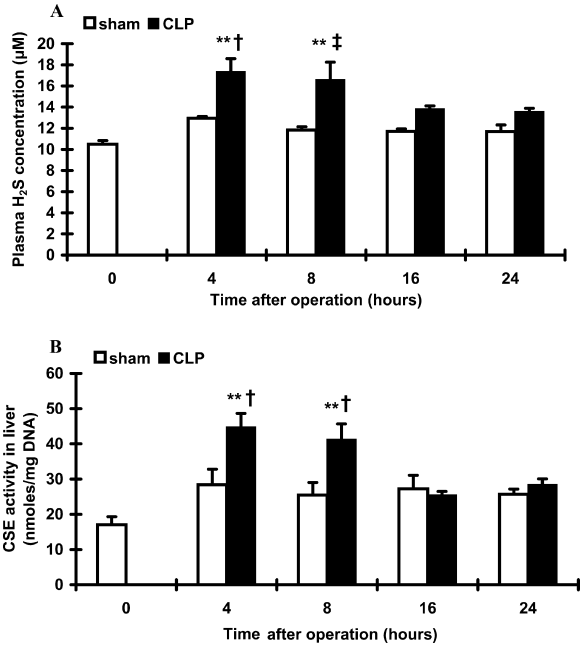

FIGURE 1. Time course study of plasma H_2S level (*A*) and liver CSE activity (*B*) in CLP-induced sepsis. Male Swiss mice were subjected to CLP or sham operation. At indicated time points (4, 8, 16, and 24 h after CLP or sham operation), mice were sacrificed by an i.p. injection of a lethal dose of pentobarbitone. At 0 h, normal mice were used as control. Results shown are the mean ± SEM (n = 10–12 animals in each group). **, Indicates $p < 0.01$ when mice subjected to CLP were compared with normal mice. †, Indicates $p < 0.05$ when septic mice were compared with sham-operated mice. ‡, Indicates $p < 0.01$ when septic mice were compared with sham-operated mice.

Chemokine and cytokine analysis

For the measurement of cytokines (IL-1β, IL-6, and TNF-α) and chemokines (MCP-1, MIP-2) in homogenized liver and lung, ELISA kits from R&D Systems were used according to the manufacturer's instructions. The lower limits of detection of the levels of IL-1β, IL-6, TNF-α, MCP-1, and MIP-2 were 15.625, 15.625, 31.25, 3.91, and 15.625 pg/ml, respectively. The ELISA for the three cytokines and two chemokines gave reproducible results, with interassay variability of less than 9.5% and intraassay variability of less than 6.5%. Results were then corrected for the DNA content of the tissue samples (24) and were expressed as pg/μg DNA.

Preparation of nuclear extract and determination of NF-κB activation

Nuclear extracts from lung (50 mg) and liver (100 mg) were prepared by using a nuclear extraction kit, as described by the manufacturer (Active Motif). Protein concentrations in nuclear extracts were determined using Bradford assay (Bio-Rad). To monitor NF-κB activation in lung and liver tissues, we used a TransAM NF-κB p65 transcription factor assay kit (Active Motif). The kit consists of a 96-well plate, into which oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) is bound. The active form of NF-κB in the nuclear extract specifically binds to this consensus site and is recognized by a primary Ab specific for the activated form of p65 of NF-κB. A HRP-conjugated secondary Ab provides the basis

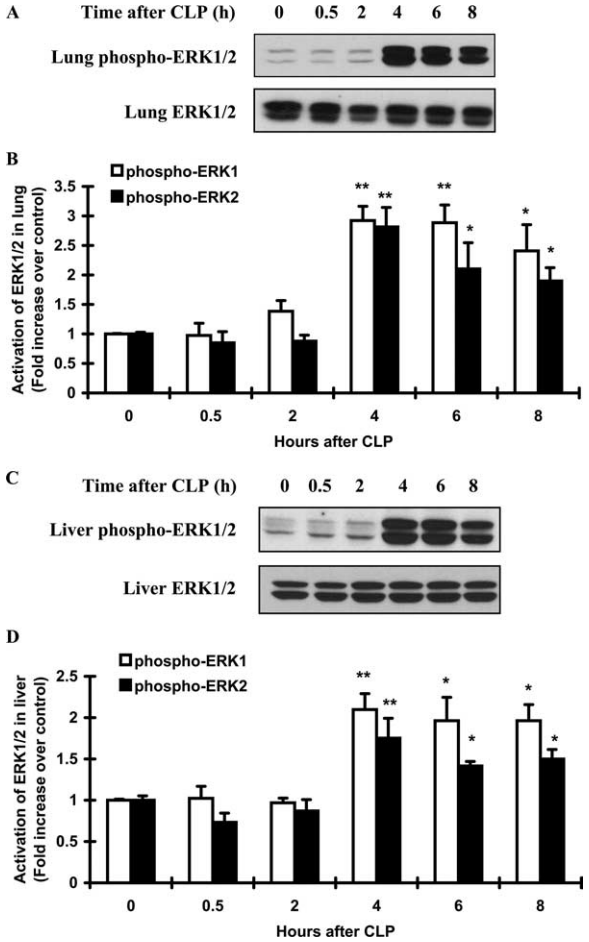

FIGURE 2. Time course study of ERK1/2 activation in lung (*A* and *B*) and liver (*C* and *D*) in CLP-induced sepsis. Male Swiss mice were subjected to CLP or sham operation. At indicated time points (0.5, 2, 4, 6, and 8 h after CLP), mice were sacrificed by an i.p. injection of a lethal dose of pentobarbitone. At 0 h, normal mice were used as control. Lung and liver were harvested and examined by Western blot for total and phospho-ERK1/2. After analysis by densitometry, the data were expressed as ratios of phosphorylated protein to total protein (plotted as fold increase over control at 0 h). Results shown are the mean ± SEM ($n = 6$ animals in each group). ∗, Indicates $p < 0.05$ when mice subjected to CLP were compared with control group at 0 h. ∗∗, Indicates $p < 0.01$ when mice subjected to CLP were compared with control group at 0 h.

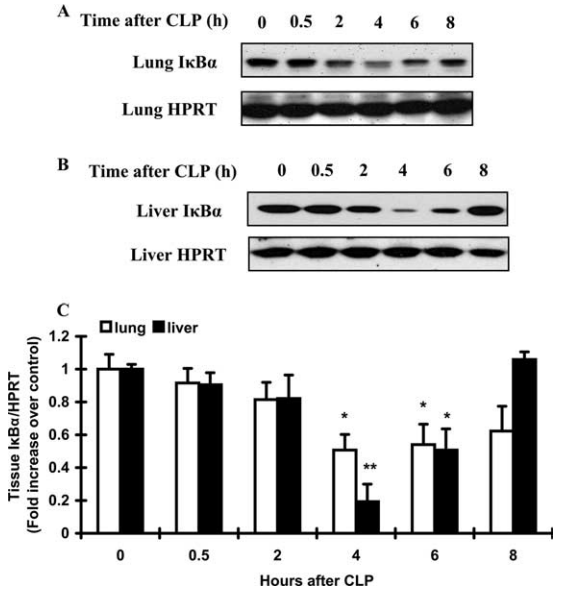

FIGURE 3. Time course study of degradation of IκBα in lung (*A* and *C*) and liver (*B* and *C*) in CLP-induced sepsis. Male Swiss mice were subjected to CLP or sham operation. At indicated time points (0.5, 2, 4, 6, and 8 h after CLP), mice were sacrificed by an i.p. injection of a lethal dose of pentobarbitone. At 0 h, normal mice were used as control. Lung and liver were harvested and examined by Western blot for IκBα and HPRT. After analysis by densitometry, the data were expressed as ratios of IκBα to HPRT (plotted as fold increase over control at 0 h). Results shown are the mean ± SEM ($n = 6$ animals in each group). ∗, Indicates $p < 0.05$ when mice subjected to CLP were compared with control group at 0 h. ∗∗, Indicates $p < 0.01$ when mice subjected to CLP were compared with control group at 0 h.

for the colorimetric quantification. The absorbance of the resulting solution was measured 2 min later (450 nm with a reference wavelength of 655 nm), using a 96-well microplate reader (Tecan Systems). The wild-type consensus oligonucleotide is provided as a competitor for NF-κB binding to monitor the specificity of the assay. Results were expressed as fold increase over the control group.

Western immunoblot

Animals were sacrificed 4 h after operation with a lethal dose of pentobarbitone (90 mg/kg), and lung and liver tissues were harvested. Lung (50 mg) and liver (100 mg) tissues were homogenized at 4°C in 0.75 ml of radioimmunoprecipitation assay lysis buffer supplemented with protease inhibitor mixture (Roche) and phosphatase inhibitor mixture (Sigma-Aldrich). The tissue homogenates were centrifuged at 14,000 × *g* for 10 min at 4°C. Protein concentration in the soluble fraction was determined by Bradford method. Protein samples (50–100 μg) were separated by SDS-PAGE on Novex 4–20% Tris-glycine polyacrylamide gels (Invitrogen) and transferred onto polyvinylidene difluoride membranes (Invitrogen) by electroblotting in Novex transfer buffer (Invitrogen) containing 20% (v/v) methanol. Membranes were then washed, blocked, and probed overnight at 4°C with rabbit anti-IκBα, phospho-IκBα, ERK1/2, phospho-ERK1/2 Abs (Cell Signaling Technology; IκBα, 1/4000; others, 1/500-1000 dilution), respectively, followed by secondary Ab for 2 h with a 1/2000 dilution of HRP-conjugated, goat anti-rabbit IgG (Santa Cruz Biotechnology). Membranes were washed and then incubated in SuperSignal West Pico chemiluminescent substrate (Pierce) before exposure to x-ray films (CL-XPosure; Pierce). Gels were calibrated by protein kaleidoscope standards (Bio-Rad). Hypoxanthine-guanine phosphoribosyltransferase (HPRT; 1/2000 dilution; Santa Cruz Biotechnology) was applied as an internal control to normalize protein loading. The intensity of bands was quantified using LabWorks Image Analysis software (Ultraviolet Products).

Alanine aminotransferase (ALT) assay

Plasma ALT activity was measured using a kinetic spectrophotometric assay (Infinity ALT reagent; Thermo Environmental Instruments), according to the manufacturer's instructions.

Statistics

The data were expressed as mean ± SEM. The significance of differences among groups was evaluated by ANOVA with posthoc Tukey's test when comparing three or more groups. The significance of differences between two groups was evaluated by Student's *t* test. The overall survival analysis for lethal injury was described by Kaplan-Meier plots. The difference of mortality among groups was tested by log rank test. A $p < 0.05$ was regarded as statistically significant.

Results

Changes over time of plasma H_2S concentration and liver CSE activity in sepsis

To imitate the clinical scenario of sepsis, mice were subjected to CLP, an operation that causes peritonitis. After CLP, plasma H_2S concentration increased in a time-dependent manner and peaked 4–8 h after CLP (Fig. 1*A*; $p < 0.01$, compared with normal control at both time points; $p < 0.05$, compared with sham group at both time points). In an attempt to identify the source of the elevation

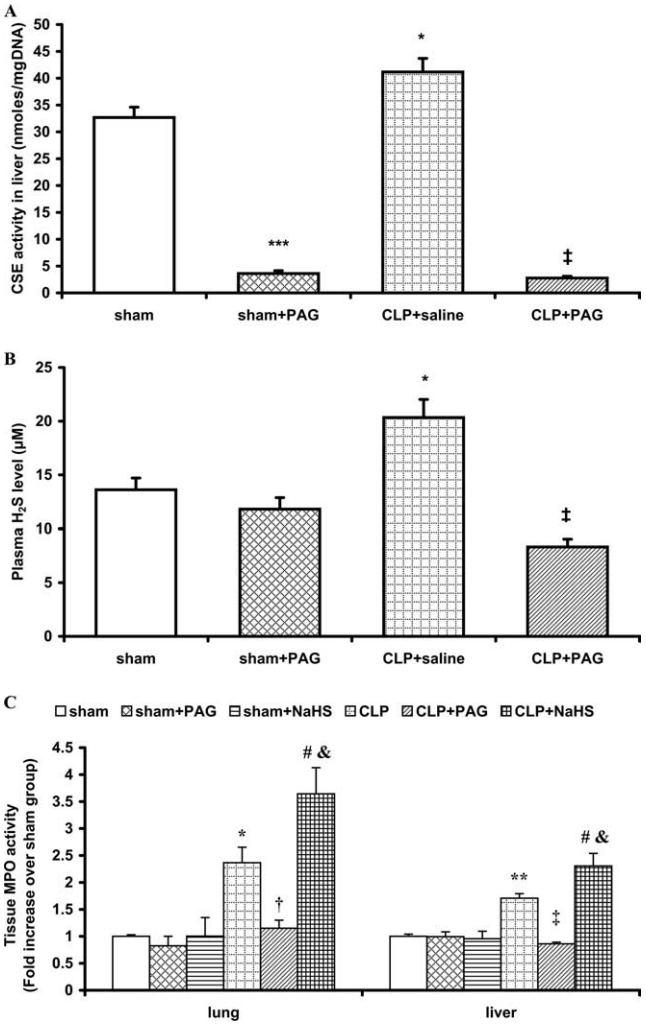

FIGURE 4. Effect of PAG administration on liver CSE activity (*A*), plasma H_2S concentration (*B*), and effect of PAG or NaHS administration on tissue MPO activity (*C*) in CLP-induced sepsis. PAG (50 mg/kg, i.p.) were given to mice 1 h before CLP or sham operation, whereas NaHS were given to mice at the same time as sham or CLP operation. Four hours after CLP or sham operation, plasma H_2S concentration, liver CSE activity, and MPO activity in lung and liver were measured, as described in *Materials and Methods*. Results shown are the mean ± SEM ($n = 10–12$ animals in each group). ∗, Indicates $p < 0.05$ when septic mice without PAG or NaHS injection were compared with sham-operated mice with or without PAG or NaHS injection. ∗∗, Indicates $p < 0.01$ when septic mice without PAG or NaHS injection were compared with sham-operated mice with or without PAG or NaHS injection. ∗∗∗, Indicates $p < 0.01$ when sham-operated mice with PAG pretreatment were compared with mice with sham operation only. †, Indicates $p < 0.05$ when septic mice with PAG pretreatment were compared with septic mice without PAG injection. ‡, Indicates $p < 0.01$ when septic mice with PAG pretreatment were compared with septic mice without PAG injection. #, Indicates $p < 0.05$ when septic mice with NaHS injection were compared with septic mice without NaHS injection. &, Indicates $p < 0.01$ when septic mice with NaHS injection were compared with sham-operated mice with or without PAG or NaHS injection.

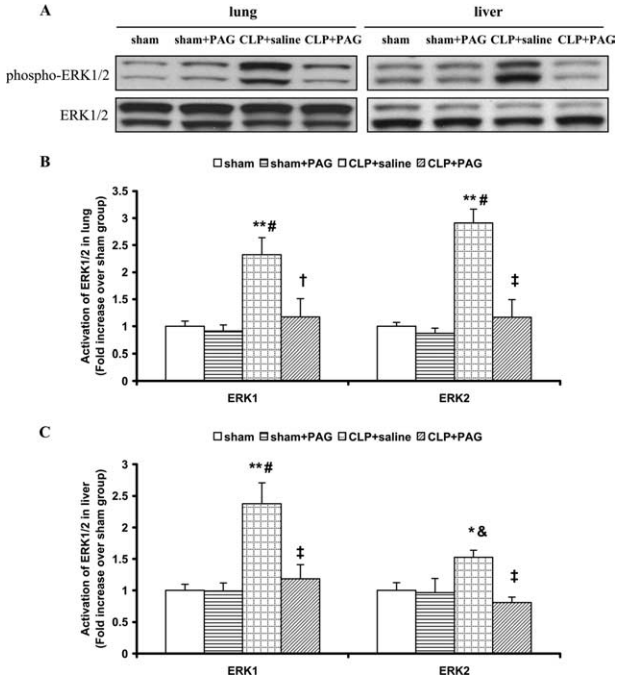

FIGURE 5. Effect of PAG administration on ERK1/2 activation in lung (*A* and *B*) and liver (*A* and *C*) in CLP-induced sepsis. Mice with CLP-induced sepsis were randomly given PAG (50 mg/kg, i.p.) or saline 1 h before CLP or sham operation. Four hours after CLP or sham operation, lung and liver were harvested and examined by Western blot for total and phospho-ERK1/2. After analysis by densitometry, the data were expressed as ratios of phosphorylated protein to total protein (plotted as fold increase over sham group). Results shown are the mean ± SEM ($n = 6$ animals in each group). ∗, Indicates $p < 0.05$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with saline injection. ∗∗, Indicates $p < 0.01$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with saline injection. &, Indicates $p < 0.05$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with PAG injection. #, Indicates $p < 0.01$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with PAG injection. †, Indicates $p < 0.05$ when septic animals with PAG pretreatment were compared with septic animals with saline injection. ‡, Indicates $p < 0.01$ when septic animals with PAG pretreatment were compared with septic animals with saline injection.

in plasma H_2S level after onset of sepsis, we examined the tissue H_2S-synthesizing activity of CSE, which is a major H_2S-forming enzyme in the cardiovascular system and highly expressed in liver and kidney (1–3, 25). It was found that CSE activity in liver, but not in kidney (data not shown), enhanced in a time-dependent manner and reached its peak 4–8 h after CLP (Fig. 1*B*). After 8 h, both liver CSE activity and plasma H_2S concentration gradually declined and returned to the comparable levels observed in normal mice or mice with sham operation. These data indicate that it is the hyperactivity of CSE in liver that leads to the overproduction of endogenous H_2S during polymicrobial sepsis. In contrast, sham operation only resulted in a slight and insignificant elevation in liver CSE activity and plasma H_2S concentration.

Phosphorylation of ERK1/2 and degradation of IκBα at early stages of sepsis

To investigate the cellular and molecular changes during sepsis, we evaluated the activation of ERK-NF-κB pathway, an important signaling transduction in sepsis (12, 13). Because a significant overproduction of endogenous H_2S was observed within 8 h after CLP, we investigated the kinetics of phosphorylation of ERK1/2 as well as degradation of IκBα at the early stages of polymicrobial sepsis. As shown in Fig. 2, an early activation of pulmonary and hepatic ERK1/2 was found, with activity reaching maximum 4 h after CLP. Furthermore, in agreement with changes over time of activation of ERK1/2, the content of IκBα in lung and liver was

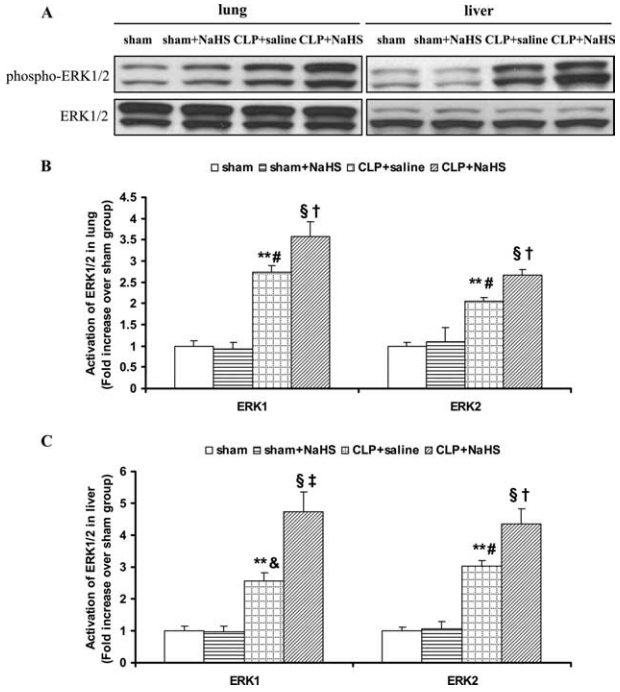

FIGURE 6. Effect of NaHS administration on ERK1/2 activation in lung (*A* and *B*) and liver (*A* and *C*) in CLP-induced sepsis. Mice with CLP-induced sepsis were randomly given NaHS (10 mg/kg, i.p.) or saline at the same time as CLP or sham operation. Four hours after CLP or sham operation, lung and liver were harvested and examined by Western blot for total and phospho-ERK1/2. After analysis by densitometry, the data were expressed as ratios of phosphorylated protein to total protein (plotted as fold increase over sham group). Results shown are the mean ± SEM (n = 6 animals in each group). **, Indicates $p < 0.01$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with saline injection. &, Indicates $p < 0.05$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with NaHS injection. #, Indicates $p < 0.01$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with NaHS injection. †, Indicates $p < 0.05$ when septic animals with NaHS injection were compared with septic animals with saline injection. ‡, Indicates $p < 0.01$ when septic animals with NaHS injection were compared with septic animals with saline injection. §, Indicates $p < 0.01$ when septic mice with NaHS injection were compared with sham-operated mice with saline or NaHS injection.

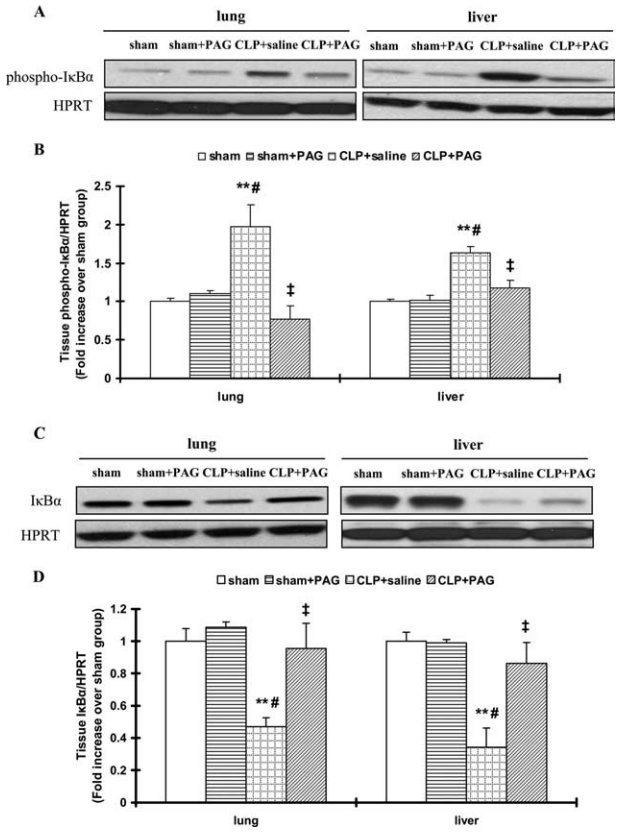

FIGURE 7. Effect of PAG administration on phosphorylation (*A* and *B*) and degradation (*C* and *D*) of IκBα in lung and liver in CLP-induced sepsis. Mice with CLP-induced sepsis were randomly given PAG (50 mg/kg, i.p.) or saline 1 h before CLP or sham operation. Four hours after CLP or sham operation, lung and liver were harvested and examined by Western blot for phospho-IκBα, IκBα, and HPRT. After analysis by densitometry, the data were expressed as ratios of phospho-IκBα or IκBα to HPRT (plotted as fold increase over sham group). Results shown are the mean ± SEM (n = 6 animals in each group). **, Indicates $p < 0.01$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with saline injection. #, Indicates $p < 0.01$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with PAG injection. ‡, Indicates $p < 0.01$ when septic animals with PAG pretreatment were compared with septic animals with saline injection.

reduced in a time-dependent manner after induction of sepsis, suggesting the occurrence of degradation (Fig. 3). The maximum degradation of IκBα in lung and liver was obtained 4 h after CLP. Because most parameters reached the peak 4 h after CLP challenge, the effect of H_2S on ERK-NF-κB pathway in polymicrobial sepsis was evaluated 4 h after CLP.

Effect of H_2S on phosphorylation of ERK1/2 in sepsis

To elucidate the effect of H_2S on ERK-NF-κB pathway in sepsis, both H_2S inhibitor and H_2S donor were applied. Pretreatment with PAG (50 mg/kg, i.p.), an irreversible blocker of CSE, 1 h before CLP almost completely abolished liver CSE activity, therefore resulting in a significant reduction in circulatory H_2S level (Fig. 4, *A* and *B*; $p < 0.01$). Plasma level of H_2S in septic mice with PAG pretreatment was comparable to that in animals with sham operation. In contrast, PAG pretreatment did not result in a further reduction in the plasma H_2S concentration in mice with sham operation, although it significantly blocked the liver CSE activity. These observations may be due to the fact that two pyridoxal-5′-phosphate-dependent enzymes, CSE and CBS, are responsible for the majority of endogenous H_2S production in mammalian tissues (1–3). Once the activity of CSE is inhibited, CBS may compensate for the loss of H_2S synthesis, and therefore maintain a relatively steady level of endogenous H_2S. As such, PAG had no obvious effect on the plasma H_2S level in sham group, and only restored the plasma H_2S concentration in septic mice to a comparable level observed in sham group, but did not eliminate the circulatory H_2S.

Before we started investigating the effect of H_2S on ERK pathway, we examined the potential role of H_2S in modulating inflammation 4 h after CLP (Fig. 4*C*). Inhibition of H_2S by pretreatment with PAG significantly decreased the sepsis-induced systemic inflammation, assessed by tissue MPO activity (a marker of neutrophil infiltration), whereas NaHS markedly increased the MPO activity in lung and liver 4 h after CLP. However, neither pretreatment with PAG nor application of NaHS had a significant effect on tissue MPO activity in sham group, indicating that H_2S may participate in regulating the magnitude of inflammatory response exclusively triggered by CLP-induced bacterial invasion of peritoneal cavity, but not the nonspecific and temporary inflammation caused by sham operation.

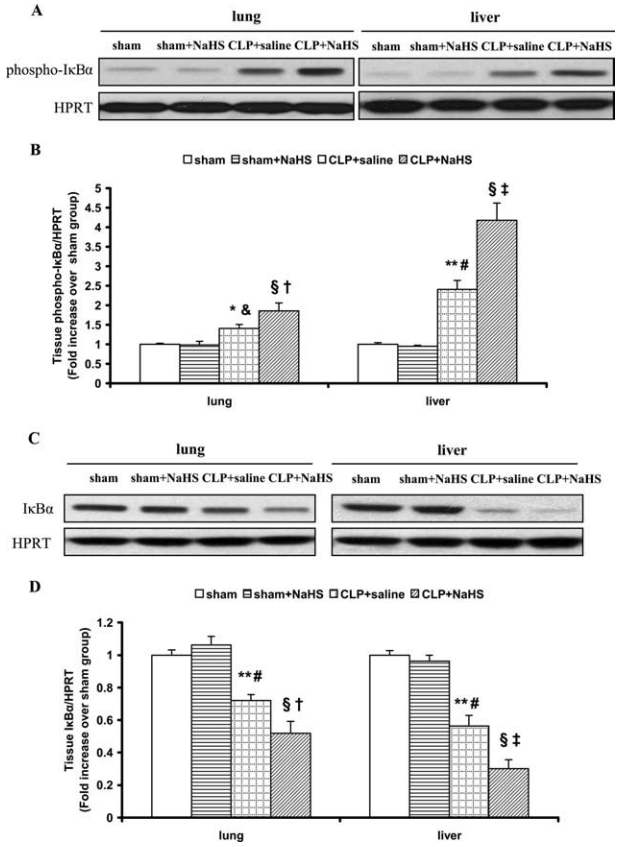

FIGURE 8. Effect of NaHS administration on phosphorylation (*A* and *B*) and degradation (*C* and *D*) of IκBα in lung and liver in CLP-induced sepsis. Mice with CLP-induced sepsis were randomly given NaHS (10 mg/kg, i.p.) or saline at the same time as CLP or sham operation. Four hours after CLP or sham operation, lung and liver were harvested and examined by Western blot for phospho-IκBα, IκBα, and HPRT. After analysis by densitometry, the data were expressed as ratios of phospho-IκBα or IκBα to HPRT (plotted as fold increase over sham group). Results shown are the mean ± SEM ($n = 6$ animals in each group). *, Indicates $p < 0.05$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with saline injection. **, Indicates $p < 0.01$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with saline injection. &, Indicates $p < 0.05$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with NaHS injection. #, Indicates $p < 0.01$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with NaHS injection. †, Indicates $p < 0.05$ when septic animals with NaHS injection were compared with septic animals with saline injection. ‡, Indicates $p < 0.01$ when septic animals with NaHS injection were compared with septic animals with saline injection. §, Indicates $p < 0.01$ when septic mice with NaHS injection were compared with sham-operated mice with saline or NaHS injection.

By Western blot analysis, we observed that inhibition of H_2S formation by pretreatment with PAG significantly suppressed the phosphorylation of ERK1/2 in lung and liver 4 h after CLP (Fig. 5). In contrast, administration of NaHS, an H_2S donor, substantially augmented tissue activation of ERK1/2 in sepsis (Fig. 6).

Effect of H_2S on degradation of IκBα and activity of nuclear NF-κB in sepsis

Phosphorylation, ubiquitination, and degradation of IκB result in NF-κB nuclear translocation, and consequently initiate the transcription of proinflammatory mediators, which play pivotal roles in immune and inflammatory response in sepsis (26). Therefore, we further examined the effect of H_2S on degradation of IκBα and

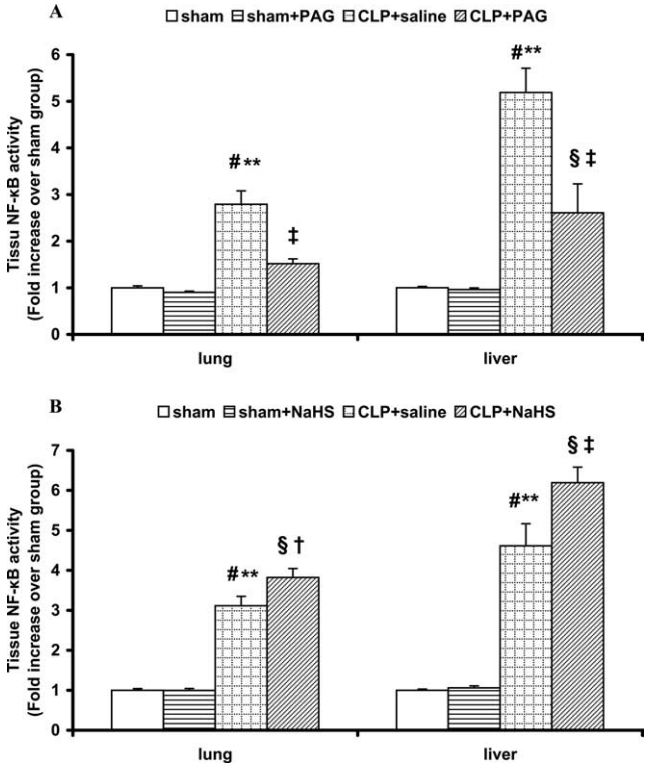

FIGURE 9. Effect of PAG (*A*) or NaHS (*B*) administration on NF-κB activation in nuclear extracts of lung and liver from mice with CLP-induced sepsis. Mice with CLP-induced sepsis were randomly given PAG (50 mg/kg, i.p.) or saline 1 h before CLP or sham operation. NaHS (10 mg/kg, i.p.) was given at the same time as CLP or sham operation. Four hours after CLP or sham operation, the DNA-binding activity of NF-κB in nuclear extracts of lung and liver was measured, as described in *Materials and Methods*. Results shown are the mean ± SEM ($n = 6–8$ animals in each group). **, Indicates $p < 0.01$ when mice subjected to CLP with saline injection were compared with animals subjected to sham operation with saline injection. #, Indicates $p < 0.01$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with PAG or NaHS injection. †, Indicates $p < 0.05$ when septic animals with NaHS injection were compared with septic animals with saline injection. ‡, Indicates $p < 0.01$ when septic animals with PAG pretreatment or NaHS injection were compared with septic animals with saline injection. §, Indicates $p < 0.01$ when septic mice with PAG or NaHS injection were compared with sham-operated mice with or without PAG or NaHS injection.

induction of NF-κB in sepsis. Pretreatment with PAG drastically reduced the pulmonary and hepatic levels of phospho-IκBα in sepsis (Fig. 7, *A* and *B*; $p < 0.05$). Consistently, the content of IκBα in lung and liver from septic mice treated with PAG was greatly enhanced in comparison with those treated with saline, suggesting an obvious inhibition of IκBα degradation (Figs. 7*C* and 10*D*; $p < 0.05$). In contrast, exogenous H_2S from administration of NaHS resulted in a further and significant rise in the degradation of IκBα in sepsis (Fig. 8). In septic mice treated with NaHS, the content of IκBα in lung and liver was significantly reduced, whereas the pulmonary and hepatic levels of phosphorylated IκBα were further enhanced (Fig. 8; $p < 0.05$, compared with septic mice treated with saline).

As a result, inhibition of endogenous H_2S formation by PAG significantly decreased the DNA-binding activity of nuclear NF-κB in lung and liver, whereas exogenous H_2S markedly enhanced the activation of NF-κB 4 h after CLP (Fig. 9; $p < 0.05$).

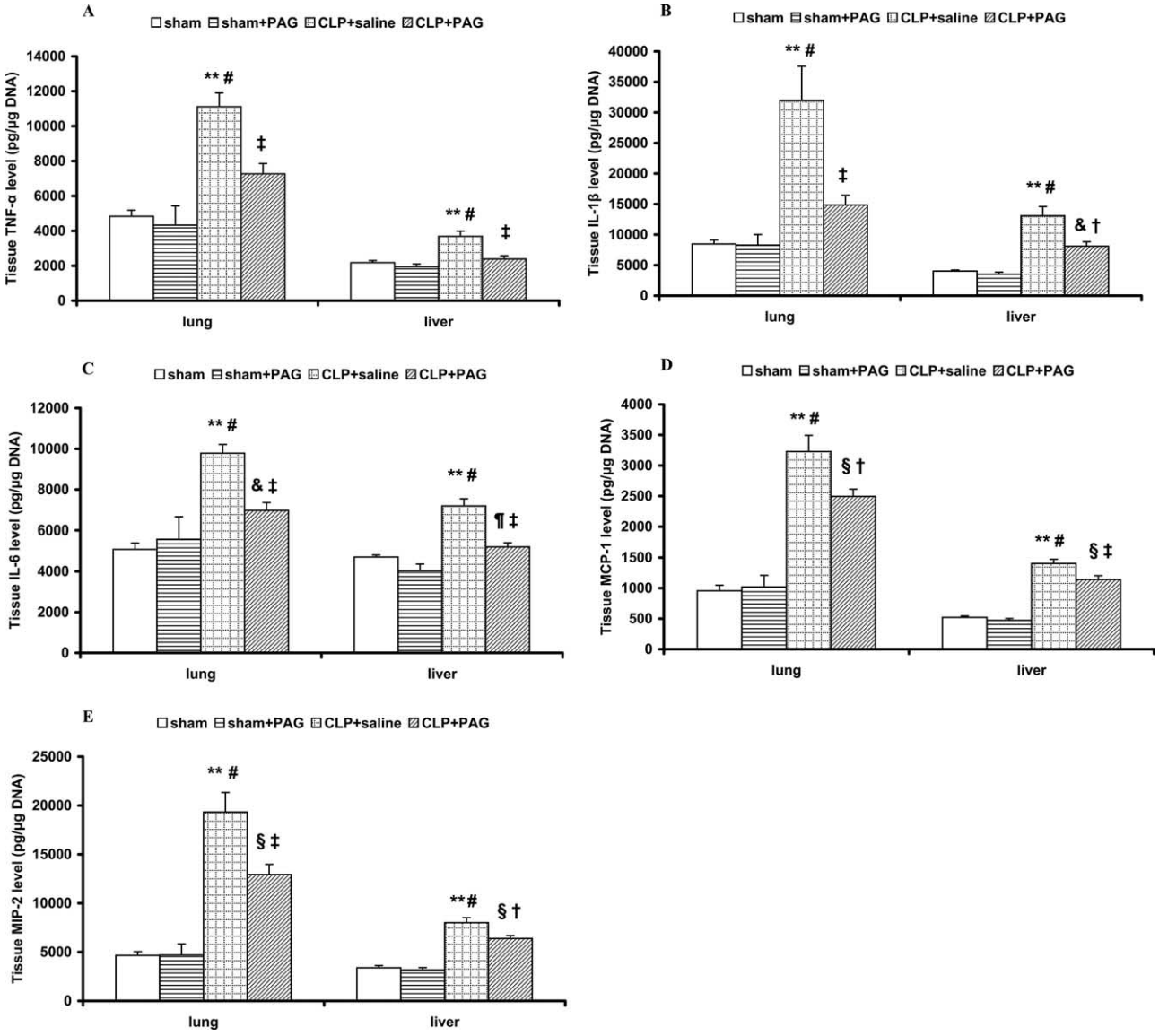

FIGURE 10. Effect of PAG administration on the tissue levels of cytokines (*A–C*) and chemokines (*D* and *E*) in CLP-induced sepsis. Mice with CLP-induced sepsis were randomly given PAG (50 mg/kg, i.p.) or saline 1 h before CLP or sham operation. Four hours after CLP or sham operation, the levels of cytokines and chemokines in lung and liver were measured, as described in *Materials and Methods*. Results shown are the mean ± SEM (n = 6–12 animals in each group). **, $p < 0.01$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with saline injection; #, $p < 0.01$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with PAG injection; †, $p < 0.05$ when septic animals with PAG pretreatment were compared with septic animals with saline injection; ‡, $p < 0.01$ when septic animals with PAG pretreatment were compared with septic animals with saline injection; &, $p < 0.05$ when septic mice with PAG pretreatment were compared with sham-operated mice with saline injection; ¶, $p < 0.05$ when septic mice with PAG pretreatment were compared with sham-operated mice with PAG injection; and §, $p < 0.01$ when septic mice with PAG pretreatment were compared with sham-operated mice with saline or PAG injection.

In addition, neither inhibition of H_2S formation by PAG nor application of NaHS affected the phosphorylation of ERK1/2, degradation of IκBα, and activation of NFκB in lung and liver in sham group (Figs. 5–9). Taken together, these findings suggest that endogenous H_2S may play an important role in regulating ERK-NF-κB-dependent signal transduction pathway in CLP-induced sepsis, but has negligible impact on nonspecific and transient inflammation due to surgical intervention.

Effect of H_2S on production of proinflammatory cytokines and chemokines in sepsis

A feature of sepsis is overproduction of proinflammatory mediators. Four hours after CLP, a substantial elevation in tissue production of TNF-α, IL-1β, IL-6, MCP-1, and MIP-2 was observed (Figs. 10 and 11). Treatment with PAG greatly suppressed the overproduction of cytokines and chemokines in lung and liver in sepsis (Fig. 10). However, treatment with NaHS amplified the tissue levels of cytokines and chemokines 4 h after CLP (Fig. 11). In addition, administration with PAG or NaHS had an insignificant effect on the tissue levels of cytokines and chemokines in sham group.

Inhibition of ERK-NF-κB pathway attenuates NaHS-induced inflammatory response in sepsis

To further confirm the association between H_2S and ERK-NF-κB pathway in sepsis, PD98059, a potent and selective antagonist of ERK kinase (MEK-1), was applied. PD98058 (10 mg/kg, i.p.; Calbiochem) or vehicle (DMSO/0.9% NaCl (1:50);

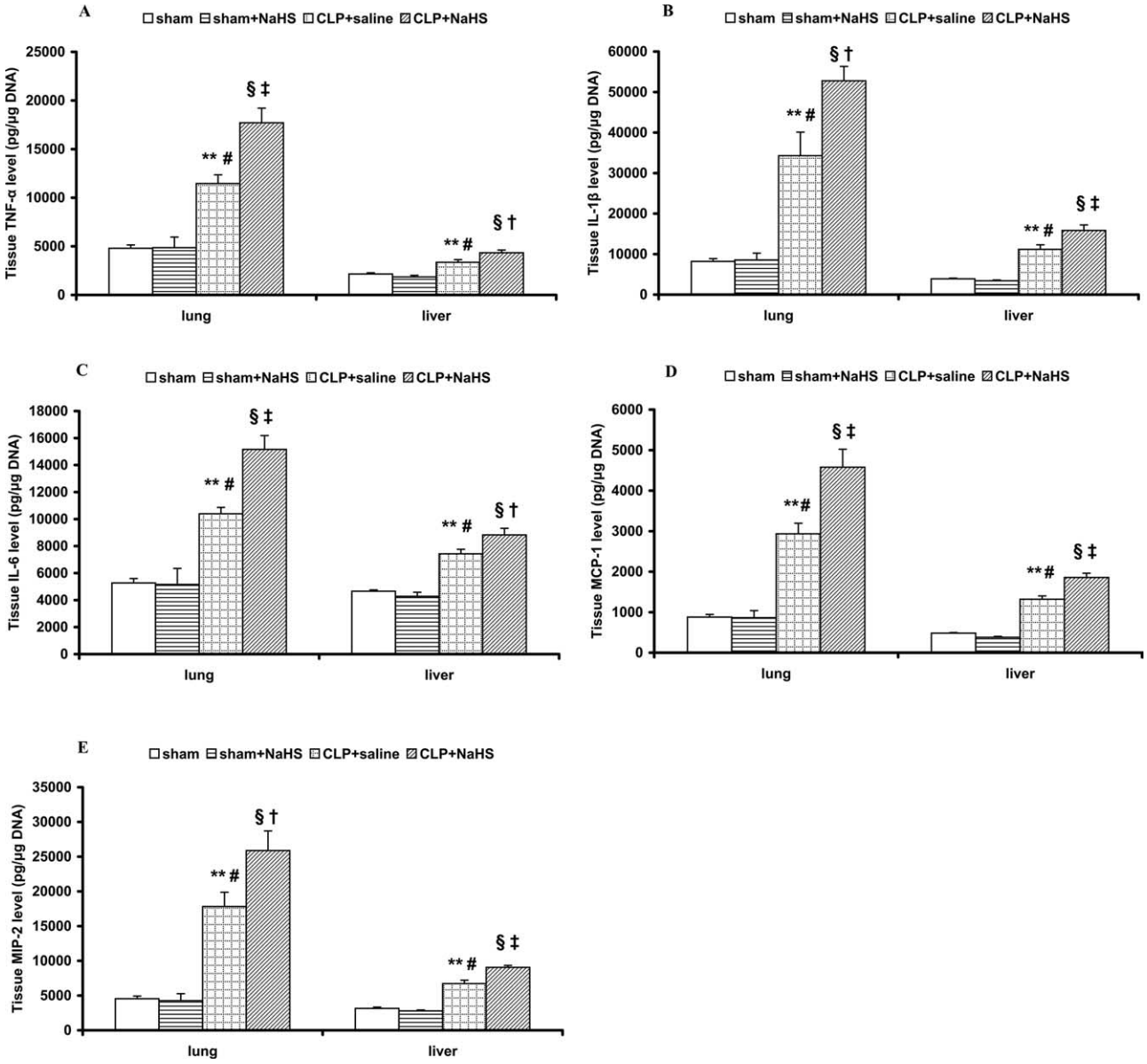

FIGURE 11. Effect of NaHS administration on the tissue levels of cytokines (*A–C*) and chemokines (*D* and *E*) in CLP-induced sepsis. Mice with CLP-induced sepsis were randomly given NaHS (10 mg/kg, i.p.) or saline at the same time as CLP or sham operation. Four hours after CLP or sham operation, the levels of cytokines and chemokines in lung and liver were measured, as described in *Materials and Methods*. Results shown are the mean ± SEM ($n = 9–12$ animals in each group). **, $p < 0.01$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with saline injection; #, $p < 0.01$ when mice subjected to CLP with saline injection were compared with mice subjected to sham operation with NaHS injection; †, $p < 0.05$ when septic animals with NaHS injection were compared with septic animals with saline injection; ‡, $p < 0.01$ when septic animals with NaHS injection were compared with septic animals with saline injection; and §, $p < 0.01$ when septic mice with NaHS injection were compared with sham-operated mice with saline or NaHS injection.

5 ml/kg, i.p.) was given to mice 1 h before CLP (19, 20). It was found that pretreatment with PD98059 at a dose of 10 mg/kg effectively suppressed the activation of ERK-NF-κB pathway in sepsis, as evidenced by a significant reduction in the phosphorylation of ERK1/2, degradation of IκBα, and DNA-binding activity of nuclear NF-κB in lung and liver (Figs. 12, 13 and 14*A*). The overproduction of proinflammatory cytokines and chemokines in sepsis was also attenuated by pretreatment with PD98059 (Fig. 14). Furthermore, pretreatment with PD98059 reduced the amplified activation of ERK-NF-κB pathway induced by NaHS in sepsis. As shown in Figs. 12, 13, and 14*A*, the phosphorylation of ERK1/2 and degradation of IκBα as well as the activation of NF-κB in lung and liver from septic mice with NaHS and PD98059 treatment were not significantly different from those with PD98059 treatment alone. As a result, PD58059 prevented NaHS from further increasing the tissue levels of cytokines and chemokines in sepsis (Fig. 14). Septic mice treated with both NaHS and PD98059 had similar levels of cytokines and chemokines in lung and liver as those treated with PD98059 alone.

Effect of H_2S on sepsis-associated hepatic dysfunction and mortality

Because the present study has proposed a key role of endogenous H_2S in systemic inflammation and the underlying signaling pathway at early stages of sepsis (4 h after CLP), it is imperative to understand whether H_2S would influence the late-phase events of sepsis, such as organ injury and death. Our

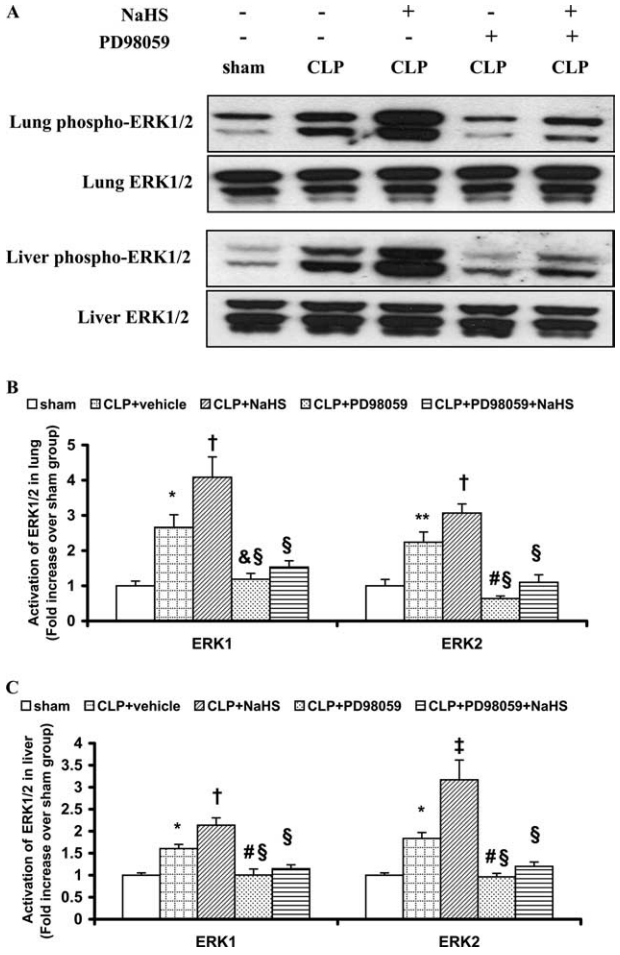

FIGURE 12. Effect of PD98059 on phosphorylation of ERK1/2 in lung (*A* and *B*) and liver (*A* and *C*) in CLP-induced sepsis. Mice were randomly given PD98059 at a dose of 10 mg/kg (i.p.) or vehicle 1 h before CLP operation. At the same time as CLP operation, mice were also given NaHS (10 mg/kg, i.p.) or saline. Sham-operated mice served as controls. Four hours after CLP or sham operation, lung and liver were harvested and examined by Western blot for total and phospho-ERK1/2. After analysis by densitometry, the data were expressed as ratios of phosphorylated protein to total protein (plotted as fold increase over sham group). Results shown are the mean ± SEM ($n = 8$–10 animals in each group). *, $p < 0.05$, septic mice with vehicle treatment compared with mice sham operation. **, $p < 0.01$, septic mice with vehicle treatment compared with mice sham operation. †, $p < 0.05$, septic mice with NaHS intervention compared with septic mice with vehicle treatment. ‡, $p < 0.01$, septic mice with NaHS intervention compared with septic mice with vehicle treatment. &, $p < 0.05$, septic mice with PD98059 pretreatment compared with septic mice with vehicle treatment. #, $p < 0.01$, septic mice with PD98059 pretreatment compared with septic mice with vehicle treatment. §, $p < 0.01$, septic mice with PD98059 pretreatment or septic mice with both PD98059 pretreatment and NaHS intervention compared with septic mice with NaHS intervention.

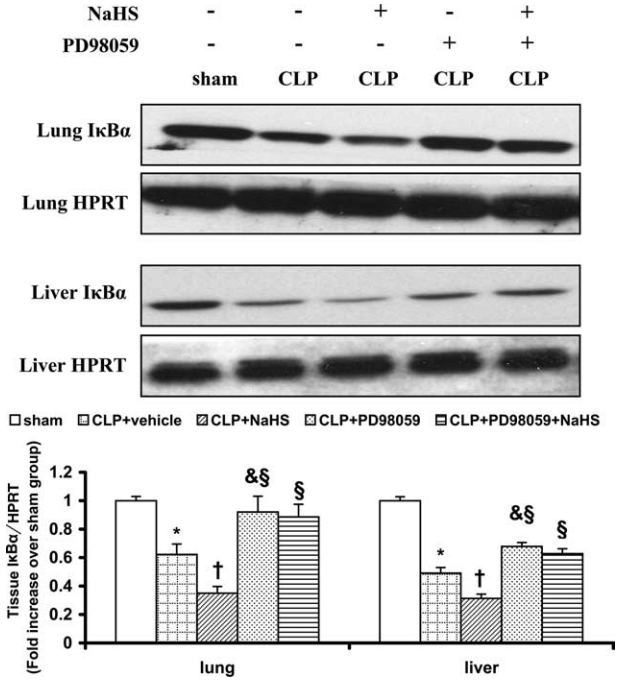

FIGURE 13. Effect of PD98059 on degradation of IκBα in CLP-induced sepsis. Mice were randomly given PD98059 at a dose of 10 mg/kg (i.p.) or vehicle 1 h before CLP operation. At the same time as CLP operation, mice were also given NaHS (10 mg/kg, i.p.) or saline. Sham-operated mice served as controls. Four hours after CLP or sham operation, lung and liver were harvested and examined by Western blot for IκBα and HPRT. After analysis by densitometry, the data were expressed as ratios of IκBα to HPRT (plotted as fold increase over sham group). Results shown are the mean ± SEM ($n = 10$ animals in each group). *, $p < 0.05$, septic mice with vehicle treatment compared with mice sham operation. †, $p < 0.05$, septic mice with NaHS intervention compared with septic mice with vehicle treatment. &, $p < 0.05$, septic mice with PD98059 pretreatment compared with septic mice with vehicle treatment. §, $p < 0.01$, septic mice with PD98059 pretreatment or septic mice with both PD98059 pretreatment and NaHS intervention compared with septic mice with NaHS intervention.

previous studies have already shown that inhibition of H_2S formation by pretreatment with PAG protects mice against lung and liver injury, and therefore improves the survival rate of sepsis (6, 7). In the present study, we found that administration of NaHS significantly exacerbated the impairment of liver function in sepsis, as evidenced by a clear increase in plasma ALT level 8 h after CLP (Fig. 15*A*). Moreover, NaHS has been suggested previously to increase lung microvascular permeability in sepsis (27). As a result, application of H_2S donor, to some extent, worsened sepsis-associated mortality, although severe and lethal CLP model was used in the present study (Fig. 15*B*; $p < 0.05$, when septic mice with NaHS were compared with septic mice without NaHS injection).

Discussion

Studies over the past several years have provided convincing evidence that H_2S acts as an endogenous modulator of inflammation in several inflammatory diseases, such as sepsis and acute pancreatitis (6–11). Preclinical animal model of sepsis as well as clinical cases of sepsis have been studied to explore the alterations of endogenous H_2S. Both CLP-induced sepsis and LPS-induced endotoxemia are associated with increased biosynthesis of endogenous H_2S. Inhibition of H_2S formation decreased systemic inflammatory response and ameliorated multiple organ damage in sepsis, suggesting a proinflammatory role of H_2S in sepsis (6–9). Plasma H_2S concentration was also significantly increased in septic patients compared with healthy control (8). In the present study, we also found a time-dependent elevation in H_2S synthesis in an animal model of polymicrobial sepsis. Our findings are consistent with the earlier observations and reinforce the importance of H_2S in sepsis. However, to the best of our knowledge, there is little information about the underlying signaling pathway by which H_2S upregulates the inflammatory response. Because various in vitro

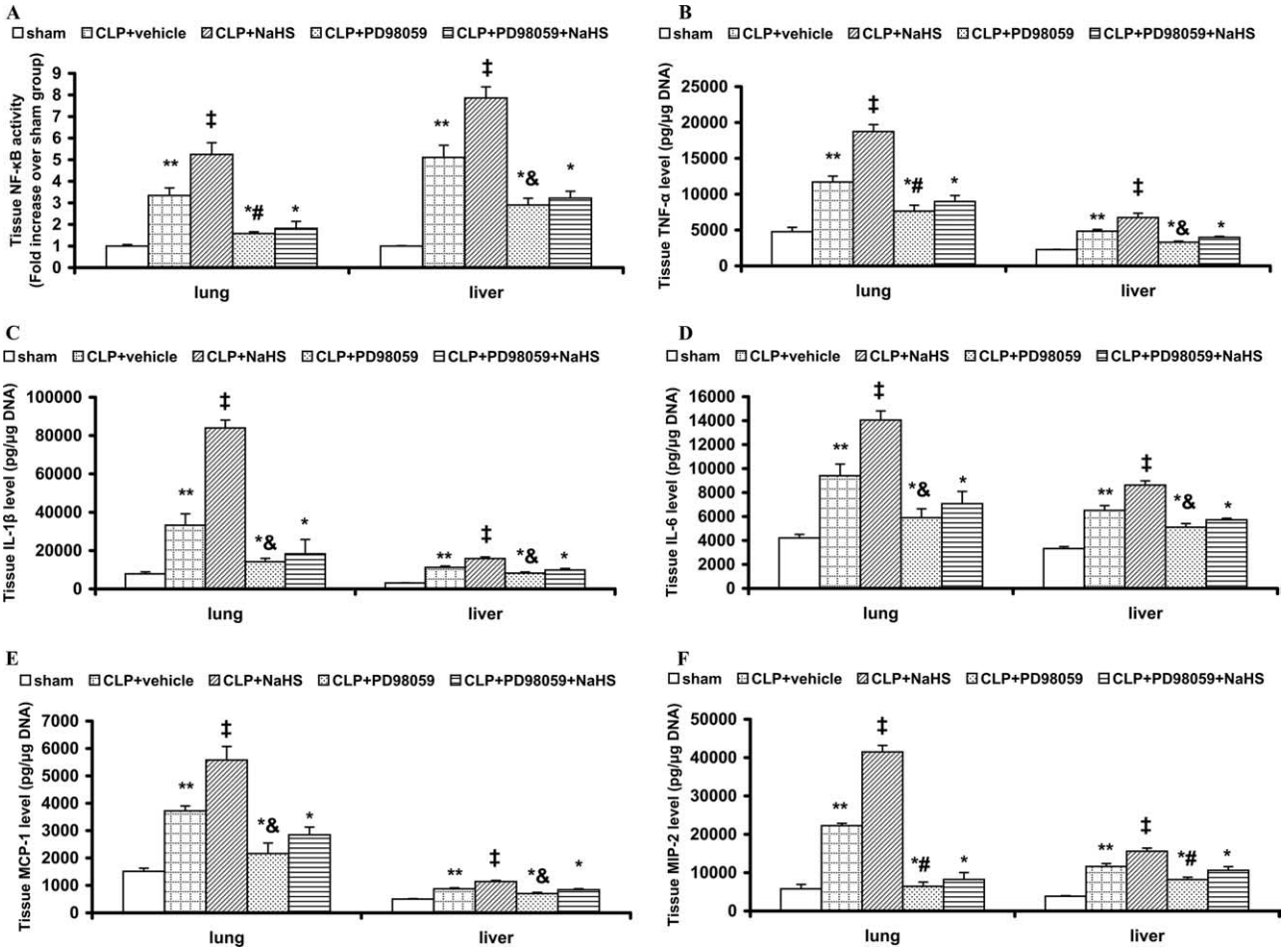

FIGURE 14. Effect of PD98059 on tissue NF-κB activity (*A*) and tissue levels of cytokines (*B–D*) and chemokines (*E* and *F*) in CLP-induced sepsis. Mice were randomly given PD98059 at a dose of 10 mg/kg (i.p.) or vehicle 1 h before CLP operation. At the same time as CLP operation, mice were also given NaHS (10 mg/kg, i.p.) or saline. Sham-operated mice served as controls. Four hours after CLP or sham operation, lung and liver were harvested and examined for tissue NF-κB activity and tissue levels of cytokines and chemokines. Results shown are the mean $\pm$ SEM ($n = 10$ animals in each group). **, $p < 0.01$, septic mice with vehicle treatment compared with mice sham operation. ‡, $p < 0.01$, septic mice with NaHS intervention compared with septic mice with vehicle treatment. &, $p < 0.05$, septic mice with PD98059 pretreatment compared with septic mice with vehicle treatment. #, $p < 0.01$, septic mice with PD98059 pretreatment compared with septic mice with vehicle treatment. *, $p < 0.01$, septic mice with PD98059 pretreatment or septic mice with both PD98059 pretreatment and NaHS intervention compared with septic mice with NaHS intervention.

and in vivo studies have suggested the significance of ERK-NF-κB pathway in inflammation, we hypothesized that this pathway may contribute to the proinflammatory role of H_2S in sepsis (28–32).

In our time course study, it was found that biosynthesis of endogenous H_2S increased in a time-dependent fashion during sepsis. Overproduction of H_2S only occurred at the early stage of sepsis and peaked 4–8 h after CLP. Similarly, pulmonary and hepatic ERK1/2 was maximally phosphorylated and activated 4 h after CLP. Furthermore, an evident peak of degradation of IκBα in lung and liver, which allows the nuclear translocation and induction of NF-κB, was also obtained 4 h after CLP. These data indicate that the temporal increase in H_2S production during sepsis seems to correlate well with the occurrence of phosphorylation of ERK and degradation of IκB. Furthermore, inhibition of H_2S formation by pretreatment with PAG significantly decreased the phosphorylation of ERK1/2, degradation of IκB, and activation of NF-κB in lung and liver during sepsis, whereas application of H_2S donor augmented them. As a result, tissue levels of inflammatory mediators during sepsis were reduced by pretreatment with PAG, but magnified by exogenous H_2S. Collectively, our data demonstrate that up-regulation of inflammatory response by H_2S in sepsis may be accompanied by the induction of NF-κB, which is dependent on the activation of ERK. However, it is important to mention that H_2S only affects the systemic inflammation and activation of ERK-NF-κB pathway induced by pathogen dissemination in CLP model rather than the nonspecific and temporary inflammatory response due to surgical procedures. This result is also consistent with the observations that sham operation did not result in an obvious alteration in plasma H_2S level and liver CSE activity. In addition, inhibition of H_2S could not completely reverse the phosphorylation of ERK1/2, degradation of IκB, activation of NF-κB, and consequent overproduction of inflammatory mediators, suggesting that other mediators rather than H_2S are involved in induction of ERK-NF-κB pathway in CLP-induced sepsis.

To directly ascertain the involvement of ERK1/2 in H_2S-induced NF-κB activation in sepsis, we used PD98059, an inhibitor of MEK-1, which is the upstream kinase of ERK1/2. Inhibition of ERK1/2 by pretreatment with PD98059 not only attenuated sepsis associated-activation of NF-κB and overproduction of proinflammatory mediators, but also abolished the amplified activation of NF-κB and enhanced generation of cytokines and chemokines

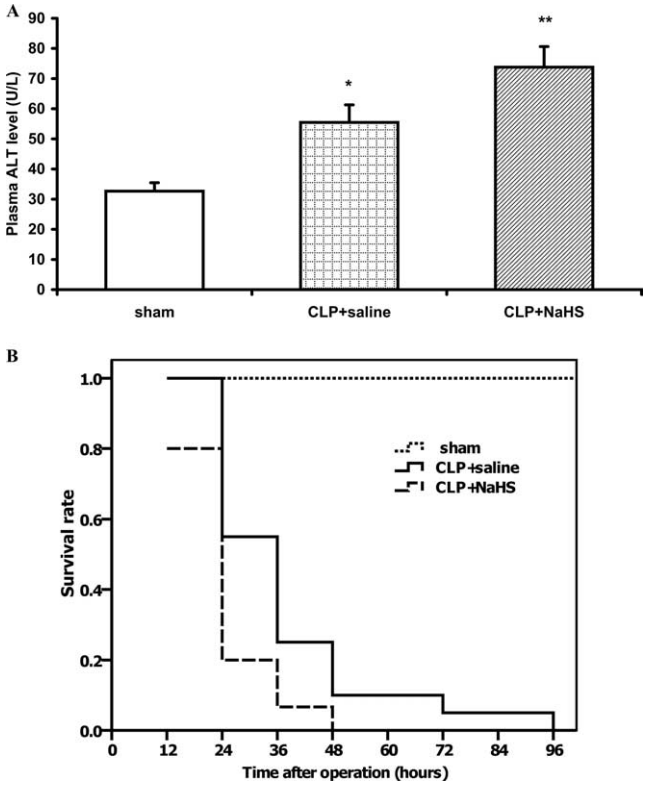

FIGURE 15. Effect of NaHS administration on sepsis-associated hepatic dysfunction (*A*) and mortality (*B*). Mice with CLP-induced sepsis were randomly given NaHS (10 mg/kg, i.p.) or saline (CLP + NaHS or CLP + saline) at the same time as CLP operation. *A*, Eight hours after CLP or sham operation, the levels of plasma ALT were measured, as described in *Materials and Methods*. Results shown are the mean ± SEM ($n = 7–8$ animals in each group). *, $p < 0.05$ when mice subjected to CLP with saline injection were compared with animals with sham operation. **, $p < 0.05$ when animals subjected to CLP with NaHS injection were compared with septic animals without NaHS injection. *B*, The survival rate was monitored for 4 days ($p < 0.05$, when septic animals with NaHS injection were compared with septic animals without NaHS injection, $n = 15–20$ in CLP + saline and CLP + NaHS group, $n = 10$ in sham group).

caused by exogenous H_2S in sepsis. These findings straightforwardly demonstrate that ERK-NF-κB signaling pathway may participate in H_2S-induced inflammation in sepsis.

Several in vitro studies have shown that H_2S induced the activation of MAPKs in various cells. For example, direct exposure of human aorta smooth muscle cells to NaHS treatment (33) as well as overexpression of CSE cDNA in HEK-293 cells (34) and human aorta smooth muscle cells (35) induced phosphorylation and activation of ERK and MAPK p38, finally contributing to cellular apoptosis. ERK and MAPK p38, but not JNK, have been reported to be involved in H_2S-induced proliferative stimulus in intestinal epithelial cells (36). In rat vascular smooth muscle cells, H_2S enhances production of NO by augmenting IL-1β-induced NF-κB activation through ERK signaling cascade (37). In immune cells, H_2S has also been suggested to stimulate the activation of human monocytes with the production of proinflammatory cytokines via ERK-NF-κB pathway (38). NaHS at a concentration of 200 μM increased the activities of ERK1/2 and p38 MAPK in RAW 264.7 macrophages (39). However, one in vitro study reported that H_2S inhibited MAPK p38 phosphorylation and caspase-3 cleavage, thus contributing to prolonged survival of human primary neutrophils (40). The discrepancy may be due to different cell types and different concentrations of NaHS used in these experiments. Taken together, these findings suggest that H_2S may regulate the phosphorylation of MAPKs and in turn contribute to different physiological and pathological roles in various types of cells and tissues.

It is to be noted that pretreatment with PD98059 could not completely abolish the amplification of NF-κB activation and subsequent inflammatory events induced by exogenous H_2S in sepsis. It suggests that in addition to ERK, H_2S may provoke NF-κB activation via other mediators. For example, H_2S causes a dose-dependent increase in intracellular calcium concentration in microglia cells, which may be mediated by cAMP/protein kinase A (PKA) (41). Because increase in intracellular Ca^{2+} and PKA facilitates NF-κB nuclear translocation and its DNA-binding activity, H_2S may modulate the activity of NF-κB and consequent inflammatory response via intracellular calcium homeostasis or PKA (42, 43). Cross-talk between H_2S and NO raises another possible way by which H_2S may interact with NF-κB in response to inflammation (37, 44). One recent study has shown that H_2S enhanced NO production and inducible NO synthase expression by enhancing IL-1β-induced NF-κB activation through a mechanism involving ERK1/2 signaling cascade in rat vascular smooth muscle cells (37). NO provided by nitroflurbiprofen reduced the biosynthesis of H_2S in LPS-induced endotoxemia, which may be due to the inhibition of transduction via the NF-κB pathway (44).

In addition, the present study provides some evidence for the potential role of H_2S in ERK-NF-κB pathway at the early stages of sepsis. It is of interest to understand the biological significance of H_2S in the events occurring at the late phases, such as multiple organ injury and death. Our previous studies together with the present study show that inhibition of H_2S formation significantly alleviates organ injury and death in sepsis, whereas NaHS aggravates sepsis-associated multiple organ damage and mortality. Therefore, it is logical to assume that endogenous H_2S may initially regulate the signal transduction (ERK and NF-κB) and subsequent production of inflammatory mediators at the early stages of sepsis, thus contributing to the pathological progression and outcome of sepsis and its sequelea (e.g., lung and liver injury).

In conclusion, our results show for the first time that H_2S may regulate inflammatory response in sepsis via the activation of ERK-NF-κB pathway. The present study may contribute to the better understanding of the precise mechanism underlying the proinflammatory role of H_2S in inflammatory diseases.

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Disclosures

The authors have no financial conflict of interest.

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