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Hydrogen Sulfide Upregulates Cyclooxygenase-2 and Prostaglandin E Metabolite in Sepsis-Evoked Acute Lung Injury via Transient Receptor Potential Vanilloid Type 1 Channel Activation

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Hydrogen sulfide (H2S) has been shown to promote transient receptor potential vanilloid type 1 (TRPV1)-mediated neurogenic inflammation in sepsis and its associated multiple organ failure, including acute lung injury (ALI). Accumulating evidence suggests that the cyclooxygenase-2 (COX-2)/PGE2 pathway plays an important role in augmenting inflammatory immune response in sepsis and respiratory diseases. However, the interactions among H2S, COX-2, and PGE2 in inciting sepsis-evoked ALI remain unknown. Therefore, the aim of this study was to investigate whether H2S would upregulate COX-2 and work in conjunction with it to instigate ALI in a murine model of polymicrobial sepsis. Polymicrobial sepsis was induced by cecal ligation approach for the management of sepsis and sepsis-associated ALI. Lethality. The strong anti-inflammatory and antiseptic actions of selective COX-2 inhibitor may provide a potential therapeutic strategy. Mice were administrated with parecoxib, a selective COX-2 inhibitor, 20 min post-CLP and subjected to ALI and survival analysis. H2S augmented COX-2 and PGE2 production in sepsis-evoked ALI by a TRPV1 channel-dependent mechanism. COX-2 inhibition with parecoxib attenuated H2S-augmented lung PGE2 production, neutrophil infiltration, edema, proinflammatory cytokines, chemokines, and adhesion molecules levels, restored lung histoarchitecture, and protected against CLP-induced lethality. The strong anti-inflammatory and antiseptic actions of selective COX-2 inhibitor may provide a potential therapeutic approach for the management of sepsis and sepsis-associated ALI. The Journal of Immunology, 2011, 187: 4778–4787.

Despite significant advances in critical care, mortality of sepsis remains unacceptably high (1). The ultimate cause of death in sepsis is severe multiple organ failure. The lungs are often the first organ to succumb postsystemic insult due to the early development of acute lung injury (ALI) or its severe form, acute respiratory distress syndrome (ARDS) (2). The underlying mechanism is thought to involve excessive liberation of inflammatory mediators that incite and perpetuate pulmonary inflammatory cascade, leading to remote ALI/ARDS (1, 3). Therefore, identification of these inflammatory molecules in the septic lungs may suggest new treatment modalities for sepsis-associated respiratory failure.

In recent years, H2S has been implicated in various inflammatory disorders including sepsis (4). H2S is synthesized endogenously from L-cysteine by cystathionine-β-synthase in the CNS and cystathionine-γ-lyase (CSE) in the cardiovascular system (5). Previously, we have reported that H2S promotes transient receptor potential vanilloid type 1 (TRPV1)-mediated neurogenic inflammation in sepsis (6). Specifically, TRPV1 is a nonselective cation channel found predominantly in primary sensory (dorsal root, trigeminal, and vagal ganglion) neurons. These sensory nerves are of mixed type composed of small-diameter, unmethylated sensory C-fibers that are most abundant, also with myelinated Aδ-fibers. Activation of TRPV1 results in opening of channel pores and influx of cations, followed by membrane depolarization with consequent release of sensory neuropeptides such as substance P (SP) that initiates the cascade of neurogenic inflammation (7).

Intriguingly, several reports have suggested a significant role for cyclooxygenase-2 (COX-2), an important inflammatory enzyme, and its most abundant and potent enzymatic product PGE2 (8) in ALI/ARDS and sepsis (9–13). Some studies suggest that COX-2 and PGE2 might be associated with SP-related inflammatory response (14–17). Unlike COX-1 that is constitutively expressed, COX-2 is an inducible enzyme stimulated by inflammatory and mitogenic stimuli (8) and has been shown to mediate inflammation by increasing vasodilation, vascular permeability, and edema in numerous respiratory diseases (18–20). In sepsis, it was demonstrated that septic insult activates macrophages to produce PGE2 through the induction of COX-2 (21). Administration of NS-398,
a specific COX-2 inhibitor, was found to inhibit PGE₂ production of endotoxin-treated macrophages to improve survival and restore leukocyte counts in burn septic mice (13). Similarly, in a rat endotoxin shock model, NS-398 significantly inhibited the height-
ened COX-2 gene expression and PGE₂ production in the aorta and peripheral blood leukocytes (22). Additionally, H₂S was shown to induce COX-2 gene and protein expression in human small intestine epithelial FHS 74 cells and isolated rat cardiac myocytes, respectively (23, 24). Despite these findings, describing the inflammatory roles of COX-2 and PGE₂ and the interactions among H₂S, COX-2, and PGE₂ in inciting sepsis-evoked ALI remain unknown. Therefore, the current study aimed to investigate whether H₂S can upregulate COX-2 and work in conjunction with it to instigate ALI in sepsis through a TRPV1 channel-dependent mechanism and, if so, to elucidate whether selective COX-2 inhibition attenuates H₂S-augmented lung inflammation and injury as well as lethality in sepsis.

Materials and Methods

Animal model of sepsis
All experiments were approved by the Animal Ethics Committee of the National University of Singapore and were conducted in accordance with established International Guiding Principles for Animal Research. A previously described model of cecal ligation and puncture (CLP)-induced sep-
sis was used (6). Briefly, male Swiss mice, 5 to 6 wk of age (25–30 g), were anesthetized with a mixture of ketamine and medetomidine (7.5 mg/kg i.p.) under aseptic conditions. A 1-cm midline incision was made through the skin and peritoneum of the abdomen. The cecum was exposed, ligated below the ileocecal valve without occluding the bowel passage, and then perforated at two locations with a 22-gauge needle distal to the point of ligation. Then, a small amount of cecal content was squeezed out gently to ensure persistence of the punctures. Finally, the bowel was repositioned, and the abdomen was closed in layers. Animals with sham operation served as controls and underwent the same procedure except that the ce-
cum was neither ligated nor punctured. All mice were resuscitated with 1 ml 0.9% sterile saline (s.c.) immediately after the surgery. Capsazepine (15 mg/kg s.c.; Sigma-Aldrich, St. Louis, MO), a TRPV1 antagonist, or vehicle (DMSO) was administered to mice 30 min before CLP. Parecoxib (30 mg/kg i.v.; Dynastat; Pfizer, New York, NY), a potent and selective COX-2 inhibitor (25–27), or saline was administered to mice 20 min after CLP. The dosage of capsaicin and parecoxib has been described in the literature and found to be effective in vivo (6, 14). N-propargylglycine (PAG; 50 mg/kg i.p.; Sigma-Aldrich), an irreversible inhibitor of CSE, was administered either 1 h before (prophylactic) or 1 h after (therapeutic) surgery, NaHS (10 mg/kg i.p.; Sigma-Aldrich), an H₂S donor, was given to mice during CLP. Animals were killed by a lethal dose of pentobarbazine (90 mg/kg i.p.) 8 h after the operation. Samples of lung were collected and stored at –80°C for subsequent analysis.

Measurement of COX-2 activity

Lung COX-2 activity was measured according to the manufacturer’s instruc-
tions (Cayman Chemical, Ann Arbor, MI). COX-2 activity was ex-
pressed as a percentage of total COX activity.

Measurement of PGE₂ metabolite levels

Lung PGE₂ metabolite (PGEM; 13, 14-dihydro-15-keto PGE₂) levels were determined by ELISA kits (Cayman Chemical) according to the manu-
facturer’s instructions. The results were corrected for the DNA content of the tissue sample (28) and expressed as fold increase over control.

Western immunoblot

Lung (50 mg) tissues were homogenized at 4°C in radioimmunopreci-
sation assay lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with 1% protease (product number P8340) and 1% phosphatase (product numbers P2830 and P5726) inhibitor cocktails (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 the Bio-Rad method. Protein samples (50 μg) were separated by SDS-PAGE on Novex Bis-Tris polyacrylamide gels (Invitrogen Life Technologies, Carlsbad, CA) and transferred onto polyvinylidene difluoride membranes (Invitrogen Life

Technologies) by electroblotting in Novex transfer buffer (Innvitrogen Life

Technologies) containing 20% (v/v) methanol. Membranes were then washed, blocked, and probed overnight at 4°C with rabbit polyclonal anti-
COX-2 (1:1000 dilution; Cayman Chemical), followed by secondary de-
tection for 2 h with an HRP-conjugated, goat anti-rabbit IgG (1:1000 di-
lution; Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed and then incubated in SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) before exposure to x-ray films (LC-Xposure; Pierce Biotechnology). Gels were calibrated by pro-
tein kaleidoscope standards (Bio-Rad, Hercules, CA). Hypoxanthine-
guanine phosphoribosyltransferase (HPRT; 1:1000 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 (Ultra-Violet Products, Cambridge, U.K.).

Measurement of myeloperoxidase activity

Neutrophil sequestration in lung was quantified by measuring myeloper-
oxidase (MPO) activity (6). Lung samples were thawed, homogenized in 20 mM phosphate buffer (pH 7.4), centrifuged (13,000 × g, 10 min, 4°C), and the resulting pellet was resuspended in 50 mM phosphate buffer (pH 6) containing 0.5% w/v hexadecyltrimethylammonium bromide (Sigma-
Aldrich). The suspension was subjected to four cycles of freezing and thawing and was further disrupted by sonication (40 s). The samples were then centrifuged (13,000 × g, 5 min, 4°C) and the supernatants 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 H₂SO₄, and the absorbance was measured at 450 nm. This absorbance was then corrected for the DNA content of the tissue samples (28), and results were expressed as fold increase over control.

Histopathological examination

A small portion of lung was excised and fixed with 10% neutral buffered formalin (Sigma-Aldrich), then subsequently dehydrated through a graded ethanol series, embedded in paraffin wax, and sectioned for routine histology. Sections of 5-μm thickness were stained with H&E and examined by light microscopy using a Carl Zeiss light microscope (Thornwood, New York, NY) (original magnification ×200).

Cytokines, chemokines, and adhesion molecules analysis

Single-analyte ELISA assays were performed for the measurement of cytokines (IL-1β, IL-6, and TNF-α), chemokines (MIP-1α and MIP-2), and adhesion molecules (P-selectin, E-selectin, ICAM-1, and VCAM-1) in homogenized lung tissue, according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). The lower limits of detection for the levels of IL-1β, IL-6, TNF-α, MIP-1α, MIP-2, P-selectin, E-selectin, ICAM-1, and VCAM-1 were 15.625, 15.625, 31.25, 31.25, 31.25, 62.5, and 62.5 pg/ml, respectively. The ELISA results were re-
producible with interassay variability of <9.5% and intra-assay variability of <6.5%. Results were then corrected for the DNA content of the tissue samples (28) and expressed as picograms per microgram of DNA.

Measurement of pulmonary edema

As an index of lung edema, the amount of extravascular lung water was calculated according to established techniques (29–31). Briefly, mice were killed 8 h after surgery, and blood was collected by cardiac puncture. The lungs were excised from mice, cleared of all extrapulmonary tissue, blotted, and weighed (total lung wet weight); they were then dried in an incubator for 48 h at 80°C and weighed again (total dry weight). For each animal, pulmonary edema was expressed as the ratio of total wet weight to total dry weight.

Survival study

Mortality was monitored every 24 h after sham or CLP procedure. The survival of these mice was recorded for up to 12 d. Mice that survived beyond this time point were considered as permanent survivors.

Statistics

The data were expressed as mean ± SEM. The significance of difference among groups was evaluated by ANOVA with a post hoc Tukey’s test for multiple comparisons when comparing three or more groups. The survival rate was estimated by the Kaplan–Meier method and compared by log-rank test. A p value < 0.05 was regarded as statistically significant.
Results

H$_2$S regulates COX-2 levels in septic lungs in a TRPV1 channel-dependent manner

Induction of sepsis by CLP resulted in a significant increase in lung COX-2 expression as compared with sham-injured mice (Fig. 1A). Administration of NaHS further enhanced pulmonary COX-2 expression in septic mice when compared with their vehicle control counterparts (Fig. 1A). In an attempt to investigate the interplay between H$_2$S and COX-2 with TRPV1-mediated neurogenic inflammation in sepsis, we tested the effect of a selective TRPV1 antagonist, capsazepine, on lung COX-2 levels. Our results revealed marked attenuation in pulmonary COX-2 expression with capsazepine in both septic and septic mice administered with NaHS (Fig. 1A). A similar profile was observed in lung COX-2 activity levels, with septic mice showing significant increase, whereas septic mice injected with NaHS exhibited greater elevation in levels (Fig. 1B). Notably, when these mice were treated with capsazepine, lung COX-2 activity levels were significantly alleviated (Fig. 1B). Furthermore, inhibition of endogenous H$_2$S formation by prophylactic or therapeutic administration of PAG markedly suppressed lung COX-2 expression (Fig. 2A) and activity (Fig. 2B) in untreated septic mice. Treatment of capsazepine in the same mice, however, did not significantly change the levels of PAG-mediated attenuation of COX-2 expression (Fig. 2A) and activity (Fig. 2B) in sepsis.

**FIGURE 1.** Inhibitory effects of capsazepine on upregulated expression (A) and activity (B) of COX-2 in septic lungs. Mice were randomly given NaHS (10 mg/kg i.p.) at the same time as CLP and capsazepine (Capz) (15 mg/kg s.c.) or vehicle (DMSO) 30 min before CLP. Sham mice served as controls. Eight hours after CLP or sham operation, lung COX-2 expression and activity levels were measured. A representative Western blot photograph is shown (A), with densitometry data expressed as average ratios of COX-2 to HPRT. Values are means ± SEM (n = 4–6 mice per group for COX-2 expression and n = 8–10 mice per group for COX-2 activity). *p < 0.01 versus sham, **p < 0.01 versus CLP + vehicle, †p < 0.05 versus CLP + vehicle, ‡p < 0.01 versus CLP + NaHS + vehicle.

**FIGURE 2.** Inhibitory effects of PAG, but not capsazepine, on upregulated expression (A) and activity (B) of COX-2 in septic lungs. Mice were randomly given PAG (50 mg/kg i.p.) 1 h before (Prophylactic) or 1 h after (Therapeutic) CLP and capsazepine (Capz) (15 mg/kg s.c.) or vehicle (DMSO) 30 min before CLP. Sham mice served as controls. Eight hours after CLP or sham operation, lung COX-2 expression and activity levels were measured. A representative Western blot photograph is shown (A), with densitometry data expressed as average ratios of COX-2 to HPRT. Values are means ± SEM (n = 4–6 mice per group for COX-2 expression and n = 8–10 mice per group for COX-2 activity). *p < 0.01 versus sham, **p < 0.01 versus CLP + vehicle.
The H$_2$S-augmented, TRPV1-dependent COX-2 response correlates with concurrent PGEM production following septic injury

The observed increases in lung COX-2 levels lead us to examine lung PGE$_2$ levels post-septic injury. PGE$_2$ is known to be rapidly metabolized in vivo to its 13, 14-dihydro-15-keto metabolite; therefore, intact PGE$_2$ in tissues obtained from whole animals are scarce (32–34). For this reason, measurement of PGE$_2$ is necessary to provide a reliable estimate of actual PGE$_2$ production (35–37). In this study, lung PGEM levels were significantly increased in septic mice as compared with sham mice (Fig. 3A). The production of PGEM was further enhanced when exogenous NaHS was administered (Fig. 3A). Importantly, treatment with capsazepine abrogated lung PGEM levels in these mice (Fig. 3A). Blockade of endogenous H$_2$S synthesis further substantiated the inflammatory role of H$_2$S in sepsis, with PAG-treated septic mice showing marked alleviation in PGEM levels, and demonstrated that this reduction in PGEM production remained unchanged in the presence of capsazepine (Fig. 3B).

COX-2 inhibition prevents H$_2$S from aggravating ALI in sepsis

The clinical pathology of ALI is characterized by pulmonary edema, neutrophil infiltration with hemorrhage, and increased production of inflammatory mediators (3). Therefore, to assess the severity of COX-2-mediated ALI in sepsis, we examined the effect of a potent and selective COX-2 inhibitor, parecoxib, on lung MPO activity, which is an important index of neutrophil sequestration, and lung wet-to-dry weight ratio to determine the levels of pulmonary edema as well as histopathological examination of lung tissues to investigate the severity of lung injury. Results from MPO activity revealed significant lung neutrophil sequestration in septic mice as compared with sham controls, and this increment was further augmented in septic mice administrated with NaHS (Fig. 4A). Notably, these effects were markedly attenuated by parecoxib (Fig. 4A). Histologically, septic mice displayed characteristic signs of ALI, which included alveolar congestion, inflammatory cell infiltration, hemorrhage, septal thickening, and interstitial edema (Fig. 4B2, B3). Restoration of normal lung histology was observed in septic mice treated with parecoxib (Fig. 4, B4), as revealed in sham-operated mice (Fig. 4BB1). Likewise, lung architecture in septic mice that received NaHS intervention was damaged more than CLP alone, with marked pulmonary congestion and intense inflammatory cellular infiltrates in the septa in addition to erythrocytes originating from ruptured capillary vessels in the lung tissues (Fig. 4BB5). However, such aggravation of lung alterations was improved by parecoxib (Fig. 4BB6). Consistent with MPO and histology results, septic mice showed elevated lung wet-to-dry weight ratio, whereas treatment with parecoxib to these mice significantly alleviated pulmonary edema (Fig. 4C). Additionally, septic mice injected with NaHS demonstrated a further rise in lung wet-to-dry weight ratio compared with their vehicle control counterparts, which was again lowered with parecoxib intervention (Fig. 4C).

Blockade of H$_2$S-mediated activation of COX-2 impaired proinflammatory cytokine, chemokine, and adhesion molecule production in sepsis-induced ALI

Septic mice showed a marked rise in lung proinflammatory cytokines TNF-α, IL-1β, IL-6, chemokines MIP-1α and MIP-2 (Fig. 5A–E), and adhesion molecules P-selectin, E-selectin, VCAM-1, and ICAM-1 (Fig. 6A–D) compared with sham mice. All of these were significantly reduced by parecoxib (Figs. 5, 6). Administration of NaHS in septic mice further enhanced the production of the aforementioned cytokines, chemokines, and adhesion molecules (Figs. 5, 6). Notably, all of their levels were greatly lowered upon treatment with parecoxib (Figs. 5, 6).

Inhibition of COX-2 attenuates H$_2$S-augmented PGEM production in septic lungs

Further evidence of lung protection by COX-2 inhibition was determined by measuring the levels of PGEM in the presence of parecoxib. As expected, lung PGEM levels were significantly increased following septic injury; however, parecoxib significantly decreased PGEM production in septic lungs (Fig. 7). Consistently, blockade of COX-2 with parecoxib drastically reduced PGEM production in septic lungs even in the presence of NaHS compared with the heightened levels detected in NaHS-injected septic mice (Fig. 7).

Inhibition of COX-2 protects against H$_2$S-augmented CLP-induced lethality, but has no effect on PAG-mediated protection of mortality in sepsis

Because the current study has proposed a key role of H$_2$S-augmented COX-2 response in sepsis-evoked ALI, it is imperative to investigate whether COX-2 inhibition would influence the late-phase events of sepsis, such as CLP-induced death. Our results revealed that mice subjected to CLP-induced sepsis displayed 0% survival by day 4 as compared with sham mice that were found normal with 100% survival throughout the 12-d study.
Sepsis and its associated multiorgan failure remain a challenging global healthcare problem for both scientists and clinicians even in the modern era of critical care management. Severe sepsis, when accompanied by respiratory failure such as ALI or ARDS, continues to plague intensive care units with high mortality that has remained $>40\%$ (1, 2). Thus, identification of endogenous inflammatory molecules that mediate inflammation and injury of this often lethal complication of common human maladies represents an important goal with immediate diagnostic and therapeutic significance. Previously, we showed that the endogenously produced H$_2$S by liver CSE, the major cell types responsible for H$_2$S formation, promotes TRPV1-mediated neuroinflammation in polymicrobial sepsis (6). In this study, we report for the first time, to our knowledge, that H$_2$S upregulates COX-2 and PGEM in sepsis through TRPV1 channel activation and that COX-2 inhibition with parecoxib, a potent and selective COX-2 inhibitor, prevents H$_2$S from exacerbating ALI and CLP-induced mortality in sepsis.

PGEM is largely thought to be synthesized from arachidonic acid via the actions of COX-2. It is known that $>90\%$ of circulating PGEM is rapidly catabolized to inactive forms on the first pass in the lungs (33, 34, 38). Therefore, studies have used the measurement of PGEM as a common alternative to indicate the levels of PGEM (35, 36, 39, 40). Results clearly demonstrate that induction of sepsis by CLP resulted in a concomitant and significant overproduction of COX-2 and PGEM in the lungs. Administration of NaHS further enhanced the biosynthesis of COX-2 and PGEM, whereas PAG significantly decreased these levels. Although hepatic CSE activity and plasma H$_2$S level have been reported to be heightened in CLP-induced sepsis (6), the usage of two different yet complementary approaches in this work—exogenous administration of NaHS that serves as an H$_2$S donor and inhibition of COX-2 with parecoxib, a potent and selective COX-2 inhibitor, prevents H$_2$S from exacerbating ALI and CLP-induced mortality in sepsis.

Through the actions of COX-2 it is largely thought that PGEM is synthesized from arachidonic acid (23). An increase in gene expression for COX-2 in H$_2$S-treated human intestinal epithelial cells was also observed (24). Nonetheless, it should be underlined that the effects of PAG alone seem not to be a consequence of blood pressure changes, given that at the doses used PAG did not significantly alter systemic blood pressure (41). Moreover, although PAG significantly inhibited the activity of liver CSE in mice with sham or CLP operation, it could not cause further decrease in the circulating level of H$_2$S and had no significant effect on tissue MPO activity in sham-operated mice (42). As such, the nonspecific effects of PAG are negligible in this study.

The usage of capsazepine, a selective TRPV1 antagonist, determined that H$_2$S upregulates COX-2 and PGEM in sepsis by a TRPV1 channel-dependent mechanism. Additionally, because blockade of TRPV1 by capsazepine attenuated H$_2$S-augmented COX-2 and PGEM response but had no effect on PAG-mediated attenuation of both parameters in septic lungs, we ascertained
from our data that sepsis has a significant sensory neurogenic component that is mediated by H\textsubscript{2}S in a TRPV1-dependent manner and that H\textsubscript{2}S stimulation of TRPV1 occurs upstream of COX-2 and PGEM in sepsis.

More importantly, we were interested to know whether the H\textsubscript{2}S-augmented, TRPV1-dependent COX-2 response can induce the production of PGEM and work in conjunction with it to incite remote ALI post-septic injury. To test this hypothesis, parecoxib, a water-soluble prodrug of valdecoxib and the first COX-2–specific inhibitor used for parenteral administration (26, 27), was employed. Notably, parecoxib exerts no deleterious effects on homeostatic functions mediated by COX-1 activation at therapeutic doses in the stomach and blood (25). Our results reveal that COX-2 inhibition with parecoxib significantly attenuated pulmonary neutrophil infiltration, edema formation, production of inflammatory cytokines, chemokines, and adhesion molecules, as well as restored lung histopathology in sepsis, thereby significantly prevented the development of sepsis-evoked ALI. Moreover, the fact that exogenous administration of NaHS to septic mice showed exacerbated ALI compared with mice subjected to CLP alone and that these pathophysiologic consequences of ALI were significantly abrogated upon treatment with parecoxib confirm the notion that sepsis-evoked ALI was specifically attributable to the interplay between H\textsubscript{2}S and COX-2. Intrigued by the strong inhibitory effects of parecoxib on H\textsubscript{2}S-induced inflammation and injury in the lungs of septic mice, our results also suggest that selective blockade of COX-2 could constitute an important therapeutic target for the treatment of sepsis-evoked ALI.

FIGURE 5. Inhibitory effects of parecoxib on heightened expressions of proinflammatory cytokines and chemokines in septic lungs. Mice were randomly given NaHS (10 mg/kg i.p.) at the same time as CLP and parecoxib (30 mg/kg i.v.) or saline 20 min after CLP. Sham mice served as controls. Eight hours after CLP or sham operation, lung levels of TNF-\(\alpha\) (A), IL-1\(\beta\) (B), IL-6 (C), MIP-1\(\alpha\) (D), and MIP-2 (E) were measured. Values are means ± SEM (n = 8–10 mice per group). *p < 0.01 versus sham, **p < 0.01 versus CLP + saline, ‡p < 0.01 versus CLP + NaHS + saline.
A number of studies have suggested a significant role for COX-2 in the development of ALI (9, 10). Specifically, COX-2 levels increased concomitantly with the severity of ALI, whereas inhibition of COX-2 attenuated ALI in an acid aspiration-induced model of ALI and carrageenan-induced pleurisy model in rats (9, 43). In addition, PGE2 has been shown to be heightened in both animal models of endotoxin shock and clinical cases of sepsis (22, 44). It has been shown to exert significant immune system dysfunction in sepsis, such as vasodilation, increased vascular permeability, and generation of local and systemic inflammatory response (45). Furthermore, it has been reported that inhibition of COX restored immune alterations and improved survival caused by sepsis (13). In agreement with these findings, our results reveal marked attenuation of lung PGEM levels in both septic and septic mice administrated with NaHS upon treatment with parecoxib as compared with the elevated levels observed in the same mice without parecoxib. These observations not only support the findings that the induction of COX-2 by H2S can translate into increased production of PGEM, but also validate the use of parecoxib as a COX-2 inhibitor.

Of even greater significance, results from our work provide convincing evidence demonstrating that the H2S induction of the COX-2/PGE2 pathway is a critical component of the lethal response associated with CLP-induced sepsis in mice. Our previous studies have reported that exogenous application of NaHS to septic mice further worsened, whereas blockade of endogenous H2S synthesis by PAG protected against organ injury and death in sepsis (6). In this study, we report that inhibition of COX-2 significantly protected against sepsis-associated mortality, as reflected by prolonged survival seen in both septic and NaHS-injected septic mice that received parecoxib intervention. Consistent with these findings, absence of COX-2 rendered COX-2–deficient mice resistant to endotoxin-induced inflammation and lethality (11). Besides, our data reveal that both prophylactic and therapeutic PAG decreased mortality significantly for up to 35–45% throughout the 12-d study period. However, survival rate was not significantly different in mice treated with both PAG and parecoxib when compared with those treated with PAG only, suggesting that the improved survival rate in sepsis was likely associated with blockade of H2S synthesis. Once formation of

**FIGURE 6.** Inhibitory effects of parecoxib on heightened expressions of adhesion molecules in septic lungs. Mice were randomly given NaHS (10 mg/kg i.p.) at the same time as CLP and parecoxib (30 mg/kg i.v.) or saline 20 min after CLP. Sham mice served as controls. Eight hours after CLP or sham operation, lung levels of P-selectin (A), E-selectin (B), VCAM-1 (C), and ICAM-1 (D) were measured. Values are means ± SEM (n = 8–10 mice per group). *p < 0.01 versus sham, **p < 0.01 versus CLP + saline, ‡p < 0.01 versus CLP + NaHS + saline.

**FIGURE 7.** Inhibitory effects of parecoxib on heightened production of PGEM in septic lungs. Mice were randomly given NaHS (10 mg/kg i.p.) at the same time as CLP and parecoxib (30 mg/kg i.v.) or saline 20 min after CLP. Sham mice served as controls. Eight hours after CLP or sham operation, lung PGEM levels were measured. Values are means ± SEM (n = 8–10 mice per group). *p < 0.01 versus sham, **p < 0.01 versus CLP + saline, ‡p < 0.01 versus CLP + NaHS + saline.
endogenously produced H2S is inhibited, H2S is unable to elicit the downstream activation of TRPV1 and the subsequent upregulation of COX-2/PGE2 in sepsis. Hence, the presence or absence of COX-2 inhibition with parecoxib does not make a difference.

Cross talk between H2S and SP raises another possible way that H2S may upregulate COX-2 and PGEM in sepsis indirectly through the effects of SP. Indeed, several studies highlighted the significance of SP and COX-2 in various inflammatory states (14–17). Particularly, SP has been demonstrated to stimulate COX-2 and PGEM upregulation through the ERK–NF-κB pathway in murine burn-induced remote ALI (14). SP was also demonstrated to augment PGE2 production in lyme disease-associated inflammatory conditions in murine microglia (46) and to induce COX-2 expression and NF-κB activation in human polymorphonuclear leukocytes (16). Furthermore, SP incites COX-2 and PGE2 expression in human coticent epithelial cells and HUVECs through the activation of JAK–STAT (15) and MAPK pathways (17), respectively. Given that endogenous H2S was known to upregulate SP in sepsis-induced ALI (47) and to regulate inflammatory response in CLP-induced sepsis by activating the ERK1/2 and NF-κB pathways (42), there exists the possibility that H2S may contribute to sepsis-evoked ALI via the signal transduction pathway of H2S–TRPV1–SP–ERK1/2–NF-κB–COX-2–PGE2. Although the exact mechanistic pathway mediating H2S induction of COX-2 remains to be investigated, H2S and SP may share a common signaling cascade involving the activation of ERK and NF-κB. Yet, they may also contribute to COX-2/PGE2 response independent of each other because SP has been shown to upregulate COX-2 and PGE2 through other signaling mechanisms.

Even though our findings indicate that COX-2 and PGE2 function as proinflammatory mediators in sepsis-associated ALI, some investigators have reported a protective role for COX-2 and PGE2 following experimental lung injury (48–50). The discrepancy may be determined by many factors, including the exact nature of the stimulus, the specific cell type involved in their production, the blend of specific surface and intracellular receptors that mediate diverse cellular events, and their differential coupling to signal transduction pathways. Furthermore, PGE2 exerts both pro- and anti-inflammatory effects depending on receptor subtypes, cell population, and context of activation (51, 52). It has been reported that PGE2 inhibits the production of cytokines like TNF-α in macrophages through the PG receptor termed E prostanoid (EP) 4 and T cell proliferation through the EP2 receptor and thus would be anti-inflammatory (53). In contrast, activation of EP3 receptor by PGE2 has been identified as having a proinflammatory stimulus in ALI because it was involved in the development of pulmonary edema (54). Additionally, the profiles of COX-2–derived PG generated in an inflammatory site change during the course of inflammation as a result of lipid mediator class switching (55). PG released during the acute phase of inflammation, notably PGE2, is proinflammatory, whereas those made in the late (resolution) phase are dominated by PGF2α, PGD2, and cyclopentenone PG, which exert anti-inflammatory effects (51). It is believed that increased levels of these endogenous proresolving mediators override the proinflammatory actions of PGE2, shifting the overall balance favoring the proresolving homeostatic mechanisms in the lung (51, 52). Furthermore, COX-2–derived PGE2 is known to mediate the formation of lipoxins, a family of potent anti-inflammatory lipid mediators, in the late phase of acute inflammation (55, 56). In a murine model of acid-initiated ALI, COX-2 has been implicated to hasten resolution of lung inflammation and injury, in part by promoting the

**FIGURE 8.** Effect of parecoxib on CLP-induced mortality in septic mice (A), septic mice injected with NaHS (B), septic mice receiving prophylactic PAG (C), and septic mice receiving therapeutic PAG (D). Mice were randomly given NaHS (10 mg/kg i.p.) at the same time as CLP or PAG (50 mg/kg i.p.) 1 h before (Prophylactic) or 1 h after (Therapeutic) CLP and parecoxib (30 mg/kg i.v.) or saline 20 min after CLP. Sham mice served as controls. Survival was monitored every 24 h for up to 12 d. Results are expressed as survival rate (n = 15–20 mice per group). Significant differences are shown in the figure.
endogenous formation of lipoxin A₄ and 15-epi-lipoxin A₄ (49). Injured human bronchial epithelial cells have also been demonstrated to promote lipoxin A₄-mediated resolution of acid-triggered airway inflammation in a COX-2-dependent manner (50). Similarly, lipoxin A₄ has been proposed to be a possible protective factor in COX-2–regulated lung protection in splanchic ischemia/reperfusion-mediated ALI (48). Collectively, these studies suggest that COX-2 may have biphasic and opposing roles in the pathophysiology of inflammation in ALI, an early proinflammatory effect and a later proresorption action. Our work, which focused on the acute events of lung inflammation and injury in sepsis, reconciled with the published findings concerning the initial, proinflammatory phenotype of COX-2 in the inflammation-resolution pathway of ALI.

In conclusion, we report for the first time, to our knowledge, that H₂S augments the upregulation of COX-2 and PGE₂, which orchestrates the neurogenic inflammatory response via activation of TRPV₁ channel, and consequently contributes to lung inflammation and injury in a mouse model of sepsis-induced ALI. Additionally, inhibition of the COX-2/PGE₂ pathway in the septic lungs significantly ameliorates inflammation, injury, and sepsis-associated mortality, thereby providing a potential therapeutic approach for the prevention of ALI in sepsis.

Disclosures
The authors have no financial conflicts of interest.

References


