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H_2S Increases Survival during Sepsis: Protective Effect of CHOP Inhibition

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Sepsis is a major cause of mortality, and dysregulation of the immune response plays a central role in this syndrome. H_2S, a recently discovered gaso-transmitter, is endogenously generated by many cell types, regulating a number of physiologic processes and pathophysiologic conditions. We report that H_2S increased survival after experimental sepsis induced by cecal ligation and puncture (CLP) in mice. Exogenous H_2S decreased the systemic inflammatory response, reduced apoptosis in the spleen, and accelerated bacterial eradication. We found that C/EBP homologous protein 10 (CHOP), a mediator of the endoplasmic reticulum stress response, was elevated in several organs after CLP, and its expression was inhibited by H_2S treatment. Using CHOP-knockout (KO) mice, we demonstrated for the first time, to our knowledge, that genetic deletion of *Chop* increased survival after LPS injection or CLP. CHOP-KO mice displayed diminished splenic caspase-3 activation and apoptosis, decreased cytokine production, and augmented bacterial clearance. Furthermore, septic CHOP-KO mice treated with H_2S showed no additive survival benefit compared with septic CHOP-KO mice. Finally, we showed that H_2S inhibited CHOP expression in macrophages by a mechanism involving Nrf2 activation. In conclusion, our findings show a protective effect of H_2S treatment afforded, at least partially, by inhibition of CHOP expression. The data reveal a major negative role for the transcription factor CHOP in overall survival during sepsis and suggest a new target for clinical intervention, as well potential strategies for treatment. ***The Journal of Immunology*****, 2014, 192: 1806–1814.**

Sepsis is a systemic inflammatory response to suspected or identified infection. Severe sepsis and septic shock remain the tenth most common cause of death in intensive care units, with >210,000 deaths annually in the United States (1, 2). This high mortality demonstrates the lack of effective therapies. Therefore, a deeper understanding of the pathophysiology of this complex syndrome is necessary to develop new and more effective treatments.

The response to infection depends on the initial host–microbial interaction. Activation of the innate immune system directs eradication of the infection while limiting the inflammatory response. At the molecular level, several signal-transduction pathways are induced, as well as transcription factors, such as NF-κB, which is a master regulator of several inflammatory mediators (3).

However, a new role is emerging for endoplasmic reticulum (ER) stress signaling and the unfolded protein response (UPR) in the regulation of inflammation and innate immunity (4, 5). Specific ER stress mediators were shown to increase the inflammatory response of dendritic cells and macrophages (6–8). Additionally, signaling from the UPR intersects with major inflammatory pathways, such as JNK and NF-κB (9). The ER exerts a central role in synthesis, maturation, and trafficking of proteins. Under stressful cellular conditions, accompanied by protein misfolding, alterations in ER Ca^{2+}, or oxidative stress, the ER elicits a complex adaptive response by activation of the UPR to re-establish cellular homeostasis. Ultimately, if the ER stress is not alleviated and cellular function is compromised, apoptosis is initiated.

C/EBP homologous protein 10 (CHOP) is a transcription factor and mediator of the UPR that plays a major role in inducing apoptosis (4, 9, 10). However, evidence indicates a new role for CHOP in the inflammatory response (11–13). CHOP expression is triggered in lungs of mice injected with LPS and after hemorrhagic trauma (14, 15). This transcription factor is crucial for induction of caspase-11 (16) that is involved in maturation of pro–IL-1β through caspase-1 (17). Accordingly, genetic loss of *Chop* results in decreased LPS-induced secretion of IL-1β (14). Thus, it is plausible that CHOP plays a major role in the pathogenesis of sepsis, although direct evidence is lacking.

H_2S is a biologically active gas serving as a messenger molecule (18). H_2S is endogenously generated in many cell types, mainly from cysteine, by two enzymes: cystathionine-β-synthase and cystathionine γ-lyase (CSE). It has a pivotal role in modulating cardiovascular function, under physiologic and pathologic conditions. Mice lacking the *Cse* gene develop hypertension (19), whereas cardiac-specific overexpression of CSE protects against myocardial ischemia-reperfusion injury (20). The role exerted by H_2S in inflammation remains controversial because it was reported to have both proinflammatory and anti-inflammatory effects (18). In animal models of sepsis, both the pharmacological inhibition of

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Abbreviations used in this article: AST, aspartate aminotransferase; CHOP, C/EBP homologous protein 10; CLP, cecal ligation and puncture; CSE, cystathionine γ-lyase; ER, endoplasmic reticulum; MPO, myeloperoxidase; PGN, peptidoglycan; siCon, silencing control; siNrf2, silencing RNA against Nrf2; TN, tunicamycin; UPR, unfolded protein response; WT, wild-type.

H_2S synthesis, as well as its administration, were shown to improve survival of septic mice (21, 22).

The present investigation examined the therapeutic benefit of administration of H_2S during polymicrobial sepsis induced by cecal ligation and puncture (CLP) in mice and the mechanistic basis for this effect. Exogenous H_2S inhibited CHOP expression, and genetic deletion of *Chop* identified this transcription factor as a key molecule in the pathogenesis of sepsis.

Materials and Methods

CLP and LPS injection

CLP protocol. Male wild-type (WT) (6–8 wk old) C57BL6/J mice and CHOP-KO mice on C57BL6/J background were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were anesthetized with isoflurane via a vaporizer at 1.5–2.5 minimum alveolar concentrations. Under sterile conditions, a 1.5-cm incision was made in the lower abdominal region, and the cecum was exposed. The distal portion of the cecum was completely ligated 1 cm from the end with a 3-0 silk suture, punctured once with an 18-gauge needle, and then replaced in the peritoneal cavity. Subsequently, the peritoneal wall and skin were closed with double sutures. A 1-ml s.c. injection of sterile saline (0.9%) was administered to mice after surgery. Sham mice underwent abdominal incision and cecal exposure without ligation and puncture. After the procedure, mice had access to water and food ad libitum. Survival was monitored for 8 d, while for cytokines, CFU, and organ harvesting, animals were sacrificed after 5 or 18 h.

LPS injection. Mice were injected i.p. with 25 mg/kg LPS (from *Escherichia coli* 055:B5), and survival was monitored for 8 d. All procedures were carried out in accordance with the Johns Hopkins University Animal Care and Use Committee–approved protocols that conformed to the *Guidelines for the Care and Use of Laboratory Animals* of the National Institutes of Health.

H_2S was generated by dissolving NaHS into saline just before use (21). One hundred microliters of the solution was injected s.c. in mice 1 h before CLP (100 μmol/kg). In the H_2S posttreatment survival experiment, mice received H_2S s.c. 2 h after the induction of sepsis, and H_2S was administered again after 24 h for the following 2 d.

Bacterial counts

Peritoneal lavage was performed with 5 ml sterile PBS. Serial dilutions of peritoneal fluid and blood were plated on brain and heart infusion agar plates (Becton Dickinson, Franklin Lakes, NJ) at 37°C. Bacterial colonies were counted after 24 h.

Cytokine measurements

Animals were sacrificed at the time specified in the figures, and blood was drawn by cardiac puncture. Plasma was diluted and assessed for cytokine levels by ELISA (eBioscience, San Diego, CA).

Myeloperoxidase activity

Leukocyte accumulation in the lung was measured as previously described, with some modification (23). Briefly, lung tissue was homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide and centrifuged for 30 min at 20,000 × *g* at 4°C. An aliquot of the supernatant was incubated with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured at 405 nm. Myeloperoxidase (MPO) activity was defined as the quantity of enzyme degrading 1 μmol H_2O_2/min at 25°C (expressed as U/g weight of tissue).

Aspartate aminotransferase

Aspartate aminotransferase levels were measured as an indicator of liver damage from mouse serum using the Vet Ace Clinical Chemistry analyzer system.

Isolation of peritoneal macrophages and RAW 264.7 culture

Cells were isolated from mice as previously described (24) and plated on cover slips; nonadherent cells were washed off after 2 h. The following day, cells were stimulated with LPS (10 μg/ml), alone or in combination with H_2S (200 μM) for 18 h. RAW 264.7 mouse macrophages were cultured in DMEM medium with penicillin, streptomycin, and 10% FBS. All cells were maintained at 37°C in the presence of 5% CO_2 in a humidified incubator.

RNA extraction and quantitative PCR analysis

For measurement of mRNA levels, total RNA was extracted from cells using TRIzol Reagent (Life Technologies). RNA was reverse transcribed into cDNA using oligonucleotide $(dT)_{20}$ primers and the SuperScript III First-Strand Synthesis System (Life Technologies Corporation). The resulting cDNA was amplified using SYBR Green Real-Time PCR and detected on an ABI 7500 system (Life Technologies) with the following amplification conditions: 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 60 s. Gene expression was calculated relative to the housekeeping gene *β-actin* using the ΔΔCt algorithm (primers used: *Chop* forward: 5′-GAGCCAGAATAACAGCCGGA-3′, *Chop* reverse: 5′-CTGTCAGCCAAGCTAGGGAC-3′).

Western blotting assay

Harvested organs were flash frozen in liquid nitrogen and stored at −80°C. Tissue was homogenized in RIPA buffer with protease and phosphatase inhibitors, and protein content was determined. Immunoblotting was performed as previously described (25). Briefly, proteins were separated on a gradient SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and blocked with 5% milk buffer. Membranes were incubated with primary Ab against CHOP, Nrf2, ATF4, or AFT6 (Santa Cruz Biotechnology, Dallas, TX), activated caspase 3 (Cell Signaling Technology, Danvers, MA), or tubulin (Abcam, Cambridge, MA) overnight at 4°C. After washing, membranes were incubated with specific secondary HRP-conjugated Abs for 1 h and incubated with HRP substrate, and signal was detected with a Kodak Image Station 4000B.

Histology and immunohistochemistry

Fixed specimens were embedded in paraffin and stained with H&E for light microscopic evaluation. Activation of caspase-3 was detected by immunoperoxidase staining, using an Ab that only recognizes the activated form. CHOP expression was identified using a rabbit anti-mouse Ab against CHOP (Santa Cruz Biotechnology). Sections were counterstained with hematoxylin. Slides were viewed with an inverted microscope (Axio Observer.A1; Zeiss), and images were acquired with a Olympus DP72 color camera using CellSense software.

Peritoneal macrophages were fixed in cold methanol, and immunostaining was carried out as previously described (26). A rabbit anti-mouse Ab (against CHOP) was used as primary Ab at 4°C overnight. After washing, fluorescent-labeled secondary Ab was added for 1 h at room temperature. Cells were washed again and mounted, and fluorescence was observed with a confocal microscope (Zeiss LSM 510 Meta).

Data analysis

Data are expressed as mean ± SD, and statistical significance was determined by an unpaired *t* test with Welch correction. A p value < 0.05 was considered significant. Survival was analyzed using the Kaplan–Meier method. Statistical analyses were performed using the log-rank test.

Results

H_2S increases survival after induction of sepsis

Previous in vivo studies assessing the impact of exogenous H_2S during sepsis reported conflicting results, revealing both a protective and a deleterious effect of H_2S (21, 22). We induced sepsis by performing CLP, a widely used animal model of polymicrobial sepsis that has the advantage of closely resembling sepsis in humans (27). One group of mice was treated s.c. with NaHS (100 μmol/kg) to generate H_2S. One hour later, severe sepsis was induced by single puncture with an 18-gauge needle in the H_2S-treated and untreated groups. We carried out a mortality study, and monitored survival for 8 d. Remarkably, we found that >80% (17 of 21) of the septic mice treated with H_2S survived compared with <25% (5 of 22) of untreated septic mice ($p < 0.0001$) (Fig. 1A). Thus, adopting the same dosage and delivery of H_2S used in the study by Spiller et al. (21), we confirmed a significant increase in the survival of septic mice treated with H_2S.

To assess the potential therapeutic use of H_2S, we evaluated the efficacy of H_2S treatment after induction of sepsis. For this purpose, sepsis was induced by CLP, and H_2S was administered s.c. 2 h later. We found that H_2S was equally effective in reducing mortality when given after the induction of sepsis (Fig. 1B).

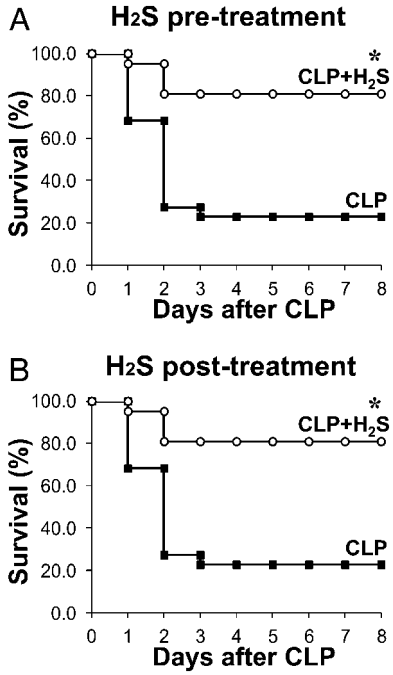

FIGURE 1. H_2S increases survival after sepsis. (**A**) Survival study of mice monitored for 8 d after CLP. A group of mice received NaHS (100 mmol/kg) as an H_2S donor s.c. (CLP+H_2S) or saline, and CLP was carried out after 1 h (CLP, $n = 21$; CLP+H_2S; $n = 22$). Data are pooled from three experiments. *$p = 0.0001$. (**B**) Sepsis was induced in mice by CLP, and H_2S was administered, as described above, 2 h later (both groups, $n = 6$). *$p = 0.01$.

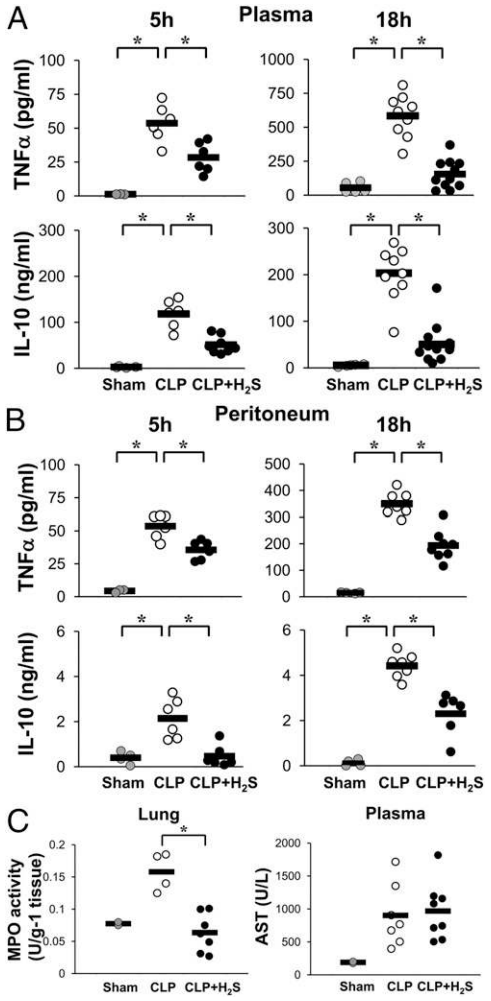

FIGURE 2. H_2S decreases inflammation. (**A**) Plasma TNF-α and IL-10 levels were measured at 5 and 18 h after CLP by ELISA (Sham, $n = 3–5$; CLP and CLP+H_2S; $n = 6–11$). Data are from individual mice from two or three experiments. (**B**) Levels of cytokines were measured in the peritoneal lavage (Sham, $n = 3–4$; CLP and CLP+H_2S; $n = 6–8$). (**C**) MPO activity from homogenized lung measured at 18 h after CLP (*left panel*). AST measured from plasma at 18 h after CLP (Sham, $n = 2$; CLP and CLP+H_2S, $n = 4–8$) (*right panel*). Data shown are from individual mice from two experiments. *$p \leq 0.05$.

H_2S reduces inflammation

Given the relevance of cytokines in regulating the inflammatory response (28, 29), we next evaluated the levels of the proinflammatory TNF-α and the anti-inflammatory IL-10 in the plasma of septic mice by ELISA. Blood levels of TNF-α were increased 5 h after CLP, as expected. H_2S treatment attenuated the increase by ~50% (Fig. 2A). TNF-α levels continued to rise 18 h after CLP to 10 times the 5-h level, and H_2S blunted this increase. Also, plasma levels of IL-10 were elevated 5 h after CLP, with a further modest increase at 18 h. H_2S attenuated the increase in IL-10 levels, as well. Then, we determined the concentration of these mediators in the peritoneum, the site of infection. A peritoneal lavage was carried out, and TNF-α and IL-10 levels were determined at 5 and 18 h post-CLP. Similar patterns of change in the levels of these cytokines were found in the peritoneal cavity (Fig. 2B) as in the plasma. Thus, H_2S treatment significantly lowered both TNF-α and IL-10 levels in the blood and at the infected site.

Then, we assessed MPO activity as a measure of neutrophil infiltration in the lung 18 h after CLP induction (Fig. 2C, *left panel*). Septic mice showed a significant increase in MPO activity in the lung compared with sham animals. Treatment with H_2S lowered this activity to basal levels. In addition, we assessed liver function by determining aspartate aminotransferase (AST) levels (Fig. 2C, *right panel*). We found that CLP increased the level of AST compared with sham; however, H_2S treatment did not prevent this increase.

Apoptosis is decreased in septic H_2S-treated mice

Next, we evaluated morphologic changes in the lung, liver, and spleen using H&E staining at 18 h after CLP. The histological sections of lung from septic mice showed signs of injury, including thickening of the alveolar septa and interstitial edema (Supplemental Fig. 1, *upper panels*). In the liver sections, swelling of hepatocytes and infiltrating neutrophils were observed (Supplemental Fig. 1, *middle panels*). Spleen from septic mice displayed numerous macrophages containing phagocytized karyorrhectic debris, likely from apoptotic cells in the white pulp (Supplemental Fig. 1, *bottom panels*). To verify cell death by apoptosis, we carried out immunostaining of spleen sections using an Ab to detect activated caspase-3 (Fig. 3A). We also examined caspase-3 activation in tissue extracts by immunoblotting (Fig. 3B). Caspase-3 activation was induced in the white pulp of spleen from septic mice. H_2S decreased its cleavage and prevented most of the above-described histological changes in organs due to CLP. Caspase-3 activation was also examined in lung and liver of septic mice by immunostaining of tissue sections and by Western blotting analysis. However, no sign of caspase-3 activation in lung or liver of septic mice was detected ≥18 h after CLP (data not shown).

H_2S increases bacterial clearance

Bacterial clearance is an important component in the resolution of infection. Therefore, we next evaluated the number of live bacteria in the blood and in the peritoneal cavity and how H_2S treatment

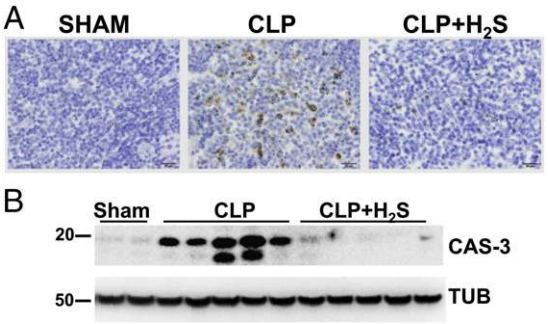

FIGURE 3. Apoptosis is reduced by H_2S treatment. (**A**) Activated caspase-3 immunoperoxidase staining was performed on spleen sections 18 h after CLP (original magnification ×400; scale bar, 20 μm). (**B**) Lysates from spleens 18 h after CLP were subjected to Western blotting assay to detect activated caspase-3 (CAS-3). Tubulin (TUB) was used as a loading control. Samples represent individual mouse spleen from two sham, five CLP, and five CLP+H_2S mice.

would modulate bacterial load (Fig. 4). H_2S did not significantly alter the number of live bacteria 5 h after CLP, either in blood or peritoneum. At 18 h, the CLP group displayed an increase in live bacteria, whereas H_2S treatment resulted in an almost complete elimination of the bacterial burden in both blood and peritoneal cavity. Thus, H_2S treatment was associated with a significantly enhanced rate of bacterial clearance.

CHOP expression is reduced by H_2S treatment in vivo

Recent findings highlighted the links between the inflammatory response and the ER stress-signaling pathways (4, 30). CHOP is emerging as a key molecule involved in the inflammatory response (11–13). Additionally, it was reported that H_2S can reduce ER stress in cardiac tissue (31) and in a neuroblastoma cell line (32). However, no study has tested the impact of H_2S on CHOP modulation during inflammation or sepsis. To elucidate the mechanism of H_2S protection, we first examined whether CHOP is expressed in vivo after CLP and whether H_2S modulates its expression. CLP-induced sepsis led to a significantly increased expression of CHOP in spleen, lung, and liver of septic mice 18 h after CLP compared with sham-operated mice (Fig. 5A). H_2S treatment in vivo was associated with decreased expression of CHOP in all of these organs.

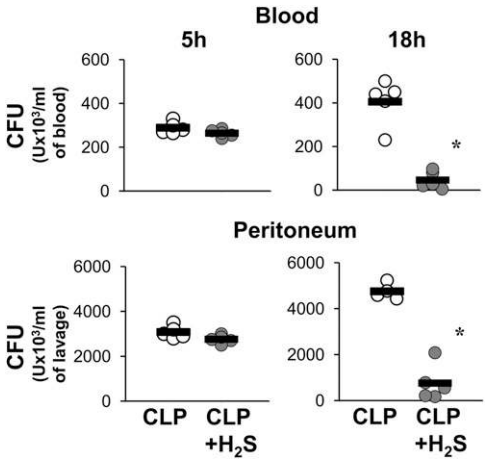

FIGURE 4. H_2S increases bacterial clearance. Whole blood (*upper panels*) and peritoneal lavage (*lower panels*) were assessed for viable bacteria by CFU assay at 5 and 18 h after CLP. Samples from single mice were streaked on brain and heart infusion agar plates for 24 h, and colonies were counted (n = 4–6). Data are a representative from two independent experiments with similar results. *$p \leq 0.05$.

Then, to investigate which cell type in the organs expressed CHOP, we performed immunostaining of tissue sections (Fig. 5B). The lungs of septic mice display CHOP expression in a disseminated pattern, probably both in inflammatory cells and lung parenchymal cells. Indeed, a previous study (14) showed that alveolar type I and type II cells express CHOP after LPS injection. In the liver, CHOP staining did not appear in hepatocytes or endothelial cells. Cells expressing CHOP are most likely tissue macrophages, such as Kupffer cells and infiltrating inflammatory cells. In the spleen, CHOP staining was evident in the sinusoidal areas outside the white pulp where macrophages mostly reside. Higher magnification shows that the CHOP$^+$ cells were large round cells with abundant cytoplasm, consistent with the morphology of macrophages. Thus, under septic conditions, CHOP was expressed mainly by inflammatory and reticulo-endothelial cells in these organs.

H_2S treatment in vivo results in downregulation of other ER stress mediators

We also investigated whether other members of the ER stress response were affected by H_2S treatment in vivo. Activation of the canonical UPR is mediated by three specific ER membrane proteins, including PERK and ATF6. Activation of the PERK pathway is implicated in attenuation of global translation initiation while increasing translation of specific transcription factors, such as ATF4, which is a main inducer of CHOP transcription. ATF6 signaling has a major role in chaperone induction and may also induce CHOP expression (9). Induction of these pathways is complex and modulates numerous proteins intersecting with a variety of inflammatory and stress signaling. In the spleen, we found that H_2S decreased CLP-induced ATF6 activation, as well as ATF4 (Fig. 6). Thus, other members of the UPR were directly or indirectly modulated by H_2S in vivo.

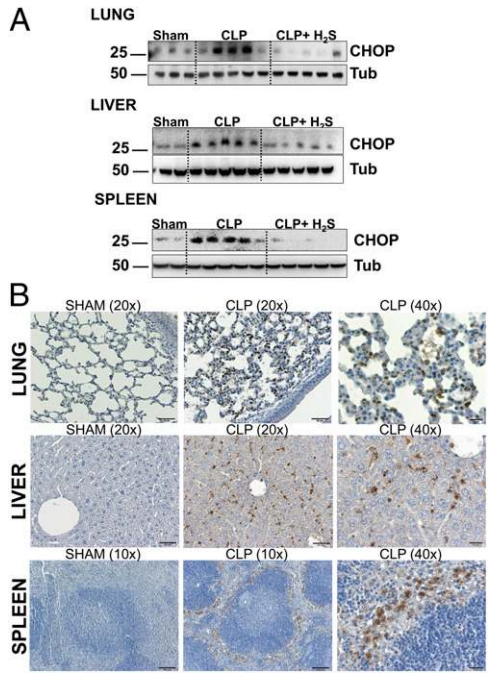

FIGURE 5. H_2S decreases CHOP expression in vivo. (**A**) Spleens, lungs, and livers harvested 18 h after CLP were homogenized and analyzed by Western blot for expression of CHOP. Tubulin (Tub) was used as a loading control. Samples analyzed are from single mice (Sham, n = 2–3; CLP and CLP+H_2S, n = 5). Data are a representative of two independent experiments with similar results. (**B**) Representative immunohistochemistry of CHOP expression in sham and CLP mice. Scale bars, 10× = 100 μm, 20× = 50 μm, and 40× = 20 μm.

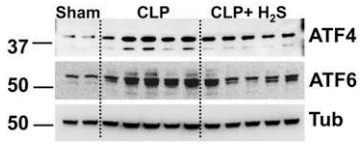

FIGURE 6. H_2S decreases other ER stress response proteins expression in vivo. Spleens harvested 18 h after CLP were homogenized and analyzed by Western blot for expression of ATF4 and ATF6. Tubulin (Tub) was used as a loading control. Samples analyzed are from single mice.

Genetic deletion of Chop *increases survival after endotoxemia or CLP-induced sepsis*

We used CHOP-KO mice to determine whether CHOP is an important mediator of the inflammatory response. A previous study (14) showed that CHOP plays a role in the pathogenesis of lung inflammation after LPS injection; however, its role in the overall outcome of mice treated with LPS has not been established. Thus, we first sought to test whether CHOP-KO mice would be more resistant to LPS-induced mortality (Fig. 7). To address this, we injected WT mice and CHOP-KO mice with LPS i.p. (25 mg/kg) and observed them for 8 d. Survival of CHOP-KO mice (87.5%) was significantly higher than that of WT mice (12.5%, $p = 0.001$).

Next, we investigated whether CHOP-KO mice are more resistant to CLP-induced sepsis. We found that septic CHOP-KO mice showed significantly improved survival compared with septic WT mice ($p = 0.01$, Fig. 8). Furthermore, we tested whether there would be an additive effect produced by H_2S treatment in combination with *Chop* genetic deletion. Treatment of septic CHOP-KO mice with H_2S did not show significant additional benefit compared with septic CHOP-KO mice ($p = 0.9$). These results clearly demonstrate that CHOP is a major negative factor in LPS- and CLP-induced mortality and provide evidence that H_2S-increased survival is mediated through CHOP inhibition.

Next, we examined the general histologic profile of liver, spleen, and lung at 18 h post-CLP in WT and CHOP KO mice (Supplemental Fig. 2). Septic CHOP KO mice displayed a lesser degree of tissue damage compared with septic WT mice, corroborating that inhibiting CHOP expression increases survival. We confirmed apoptosis by immunostaining of sections of spleen using an Ab to detect activated caspase-3 (Fig. 9). Although septic WT mice displayed activation of caspase-3, CHOP-KO mice were almost completely negative. Then, we explored whether CHOP is involved in the release of TNF-α and IL-10 in septic mice. The levels of these cytokines were significantly reduced in septic CHOP-KO mice compared with septic WT mice, both in plasma and in the peritoneal cavity (Fig. 10A, 10B). Lastly, we determined bacterial burden in CHOP-KO mice. After CLP, bacterial clearance was

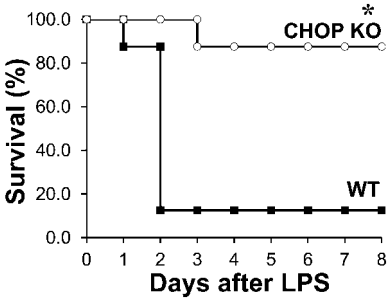

FIGURE 7. Genetic deletion of *Chop* protects against LPS-induced mortality. The survival rate of WT and CHOP-KO mice was observed for 8 d after i.p. injection of 25 mg/kg of LPS ($n = 8$, $*p = 0.001$).

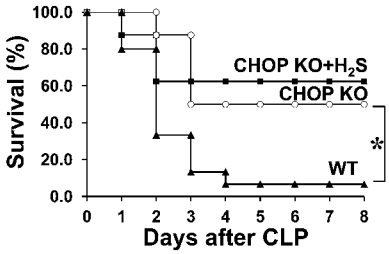

FIGURE 8. Genetic deletion of *Chop* protects against CLP-induced mortality. The survival rate of WT and CHOP-KO mice ($n = 8$ mice/group) was observed for 8 d after induction of sepsis by CLP ($n = 8$–10). A group of CHOP-KO mice also was treated with H_2S 2 h prior to CLP (CHOP KO versus CHOP KO+H_2S, $p = 0.9$). $*p = 0.01$.

markedly improved in septic CHOP-KO mice compared with WT animals, in the blood as well as the peritoneum (Fig. 10C).

Thus, genetic deletion of *Chop* increases survival after CLP by reducing the systemic inflammatory response, decreasing apoptosis, and improving bacterial clearance.

H_2S inhibits CHOP expression in macrophages by modulation of Nrf2

We used macrophages to explore the mechanism by which H_2S reduces CHOP expression, because these cells play a central role in the innate immune response. In addition, our data suggest that this cell type shows increased CHOP expression during sepsis (Fig. 5B).

First, we assessed CHOP expression and the effects of H_2S in the macrophage cell line RAW264.7 and in peritoneal mouse macrophages. RAW cells were stimulated with LPS and with constituents of Gram-positive bacterial peptidoglycan (PGN). Incubation of cells for 18 h with these inducers led to CHOP expression. H_2S treatment blunted this effect (Fig. 11A), along with a reduction in *Chop* mRNA transcript (Fig. 11B). Immunostaining of peritoneal macrophages demonstrated that LPS treatment resulted in increased CHOP expression, in agreement with a previous study (16). H_2S treatment reduced CHOP expression in primary macrophages (Fig. 11C). Next, we investigated whether H_2S inhibits the ER stress induced by tunicamycin (TN) treatment, a widely used inducer of the ER stress response. H_2S was able to reduce CHOP expression by TN (Fig. 11D). Thus, H_2S decreased CHOP expression in macrophages stimulated with bacterial products and with a direct ER stress inducer.

Subsequently, we investigated the mechanism by which H_2S decreases CHOP expression. A recent study (33) showed that the protective effect of H_2S in the heart is mediated by increased Nrf2 levels. Nrf2 is a transcription factor implicated in improving cell survival following ER stress, and it was shown to inhibit CHOP expression (34, 35). We first examined whether H_2S induces Nrf2 in macrophages. We incubated RAW cells with H_2S for up to 24 h

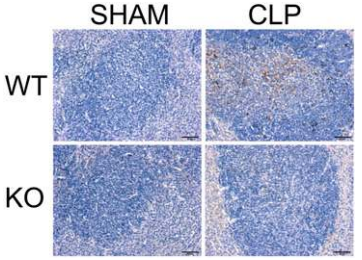

FIGURE 9. Septic CHOP-KO mice display reduced apoptosis. Representative activated caspase-3 immunoperoxidase staining was performed on spleen sections 18 h after CLP (scale bars, 50 μm).

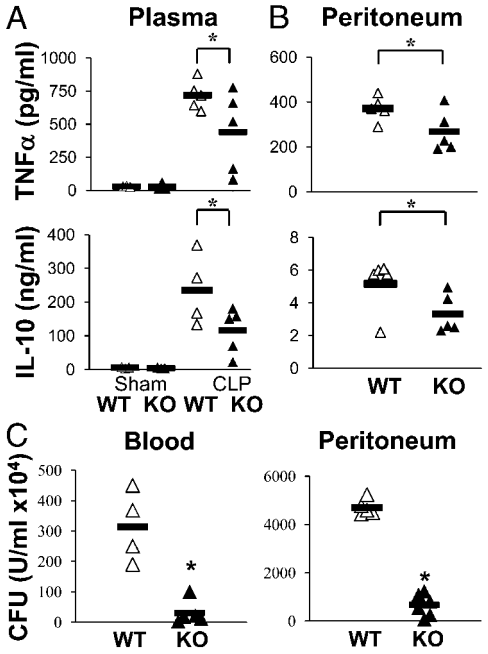

FIGURE 10. Decreased TNF-α and IL-10 and increased bacterial clearance in CHOP-KO mice. (**A**) TNF-α and IL-10 levels were measured by ELISA in the plasma of sham-operated and septic (CLP) mice from WT and CHOP-KO groups 18 h after CLP (n = 4–6). (**B**) Levels of cytokines in the peritoneum of septic WT and CHOP KO mice (n = 4–6). (**C**) CFU assay was performed on blood and peritoneum lavage at 18 h after CLP. Samples from single mice were streaked on brain and heart infusion agar plates for 24 h and then colonies were counted (n = 4–5). *$p \leq 0.05$.

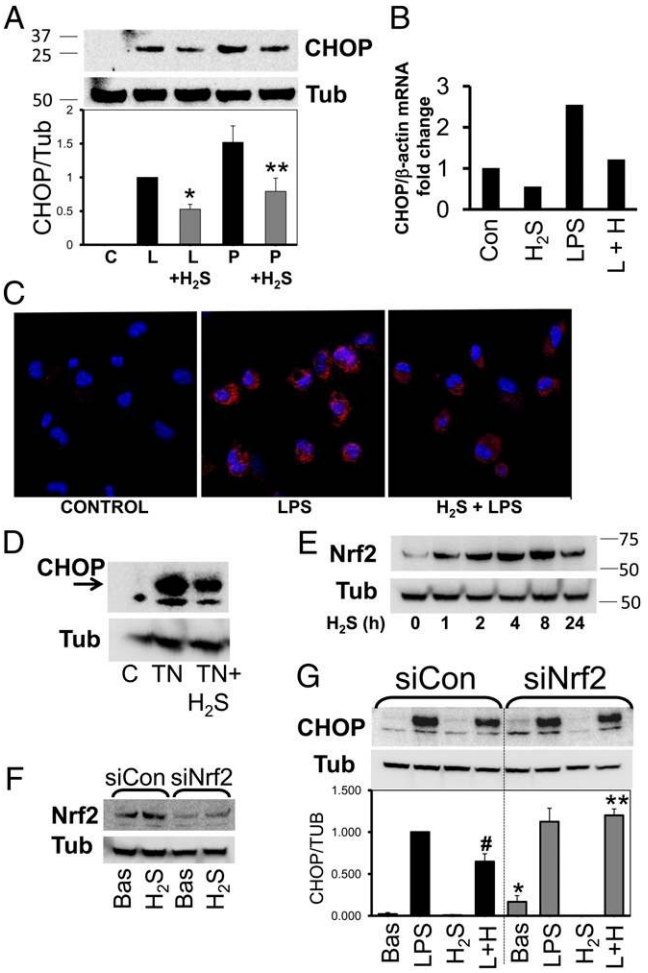

FIGURE 11. H_2S treatment decreases CHOP expression in macrophages in an Nrf2-dependent manner. (**A**) Representative Western blot of CHOP. RAW cells were incubated for 18 h with LPS (L; 10 mg/ml) or PGN (P; 10 mg/ml), with or without H_2S (200 mM). Densitometric analysis was calculated as the ratio of CHOP/tubulin (Tub) and normalized to LPS (n = 3). *$p \leq 0.01$, versus LPS alone. **$p \leq 0.01$, versus PGN alone. (**B**) CHOP mRNA is reduced after incubation of cells with LPS and/or H_2S for 6 h (n = 2). (**C**) Representative confocal image of peritoneal macrophages incubated with LPS and/or H_2S for 18 h and then stained for expression of CHOP (n = 2, original magnification ×40). (**D**) RAW cells were treated with TN for 3 h, with or without H_2S (n = 3). (**E**) Cells were incubated for the time indicated with H_2S, and Nrf2 was evaluated (n = 2). (**F**) RAW cells were transfected with scrambled oligonucleotide (siCon) or siNrf2 for 24 h and then incubated with H_2S for 18 h ($n \geq 3$). (**G**) After transfection with siCon or siNrf2, RAW cells were incubated with LPS, with (L+H) or without H_2S for 12 h. Densitometric analysis (lower panel) (n = 3). $^{\#}p \leq 0.01$, versus LPS, *$p \leq 0.01$, versus Bas, **$p \leq 0.01$, versus LPS+H_2S (L+H) of siCon group.

and assessed Nrf2 expression. Nrf2 was induced after 1 h of H_2S treatment, increased with time from 1 to 8 h, and started to decrease at 24 h (Fig. 11E).

To establish whether H_2S decreases CHOP expression through Nrf2 activation, we used silencing RNA directed against Nrf2 (siNrf2). RAW cells were transfected with a scrambled silent control (siCon) oligonucleotide or siNrf2 for 24 h and then incubated with medium (Bas) or H_2S for 18 h. Nrf2 was effectively downregulated by the siNrf2 compared with siCon (Fig. 11F). Then, we stimulated the cells with LPS in the presence or absence of H_2S. In the siCon group, LPS-induced CHOP expression was decreased by H_2S treatment. In the siNrf2 group, the basal level of CHOP was increased, and treatment with H_2S did not decrease LPS-induced CHOP expression (Fig. 11G). Our data indicate that Nrf2 modulates CHOP expression under basal conditions, and it is a major regulator of the H_2S-mediated decrease in CHOP expression.

Discussion

In this study, we report that administration of H_2S markedly improves survival of experimental polymicrobial sepsis when given prior to the onset and, more importantly, even 2 h after CLP. The improved outcome is associated with decreased cytokine production, reduced apoptosis, and increased bacterial clearance. CLP induced CHOP expression, but this effect was attenuated by H_2S treatment. We found that CHOP is a major regulator of the inflammatory response, because CHOP-KO mice are significantly resistant to LPS- and CLP-induced mortality. We also demonstrated that the protection afforded by genetic deletion of *Chop* is not affected by H_2S treatment, suggesting that CHOP may be a key mediator of H_2S-induced protection. Finally, we demonstrated that H_2S inhibition of CHOP expression is mediated by Nrf2 activation in macrophages.

Two previous investigations on the effect of exogenous H_2S on experimental sepsis led to conflicting results. Zhang et al. (22) showed that H_2S injection into septic mice resulted in increased mortality, whereas Spiller et al. (21) reported a beneficial effect. The divergent outcome of these studies may be explained by the different route of H_2S administration and the dosage used. Although a beneficial effect was observed when H_2S was administered s.c. at a dose of 100 μmol/kg (~5.6 mg/kg), an i.v. bolus (10 mg/kg) resulted in increased mortality. As the study from Spiller et al. (21) showed, an i.v. bolus of H_2S provokes a significant decrease in mean arterial pressure, whereas s.c. administration avoids this effect. The latter is likely due to its slow release into the circulation. Therefore, during sepsis, an already compromised

vascular tone could be dysregulated even further by a direct i.v. bolus of H_2S, which induces vasorelaxation in different vascular beds (18). Thus, although other factors may also have a role, our study confirms the primary importance of the route of administration in dictating whether H_2S is beneficial or deleterious in the setting of septic shock.

Our study shows that H_2S treatment resulted in decreased production of the proinflammatory TNF-α, as well as decreased leukocyte infiltration in the lungs and liver, in line with other studies (21, 36–38). Because IL-10 is known to have anti-inflammatory properties (39), we were surprised to find that H_2S treatment decreased the release of this cytokine. Previously, H_2S was reported to increase IL-10 levels in vitro and in a model of acute lung injury (37, 40), although H_2S decreased IL-10 levels during hepatic ischemic-reperfusion injury (41). It is plausible that the impact of H_2S on IL-10 levels varies, depending on the cell type or animal model used. However, and more importantly, it was shown that neutrophils from septic patients have reduced bactericidal activity when treated with IL-10, leading to impaired host defense with overgrowth or persistence of pathogens (39, 42–44). Moreover, neutralization of IL-10 in septic mice improves survival (45) and enhances phagocytosis by macrophages (46). Thus, the attenuated release of this cytokine could be important, because the overproduction of this cytokine, together with other factors, could be responsible for the immune-suppressive state that is characteristic of sepsis (47). Therefore, H_2S treatment may help to maintain an effective, but not overactive, host immune response leading to increased survival.

Another important aspect of our study is that treatment with H_2S was associated with a virtually complete inhibition of apoptosis induced by CLP. H_2S is known to decrease apoptosis in different cell types, such as neurons (48), cardiomyocytes (20), and cancer cells (49). However, this effect in a model of experimental sepsis has not been reported. Recent studies highlighted that apoptosis during sepsis plays an important role. Indeed, septic spleens from patient autopsies and from animal studies show widespread lymphocyte apoptosis during sepsis (50–55). Conversely, inhibition of lymphocyte apoptosis in experimental models leads to increased survival (53, 54, 56–58). Among the mechanisms of the anti-apoptotic effect of H_2S, the preservation of mitochondrial function through the opening of the K_{ATP} channels was shown to play an important role (18). Nevertheless, during sepsis, both the death receptor pathway and mitochondrial-mediated apoptosis have been described, as well as cross-talk between these two pathways (51, 54, 55, 57, 59). Taken together, our results suggest that the beneficial effect of H_2S is likely due to modulation of different pathways that are often interconnected, targeting apoptosis, as well as inflammatory mediators, and resulting in increased survival.

Septic mice exhibited increased expression of CHOP, and treatment with H_2S was associated with its downregulation. Although CHOP is a major inducer of apoptosis in response to ER stress (4, 9, 10), recent evidence identifies a new role for CHOP as a mediator of the inflammatory response (14–17). Our data demonstrate that this transcription factor plays a pivotal role in the pathogenesis of sepsis. Indeed, CHOP-KO mice displayed virtually no apoptosis, augmented bacterial clearance, and increased survival after CLP or LPS injection. All of the beneficial features observed in mice with genetic deletion of *Chop* were also seen in septic WT mice treated with H_2S. Furthermore, when we administered H_2S to septic CHOP-KO mice there was no additional improvement in survival compared with septic untreated CHOP-KO mice. This evidence supports the concept that suppression of CHOP may be a major factor in the beneficial effect afforded by H_2S. It is important to note that we observed no colocalization of caspase-3 activation with CHOP expression in the spleen of septic mice. Although caspase-3 activation was mainly in the white pulp of the spleen, CHOP expression was present in the outer marginal zones of this organ. Additionally, we also identified augmented CHOP expression in lung and liver; however, no detectable activation of caspase-3 was observed. Altogether, these data suggest that CHOP may not play a direct role in the apoptotic events that we observed in the spleen of septic mice, indicating that its primary role may be related to the inflammatory response per se. As shown by Endo and colleagues (16), CHOP has a critical role in the induction of caspase-11, which is needed for the production of IL-1β. In this regard, our data add new insights supporting the concept that CHOP contributes to the inflammatory response, in agreement with other studies (11–14, 16, 17). Importantly, to our knowledge, we show for the first time that genetic deletion of *Chop* leads to increased survival after CLP or LPS injection.

It is unknown whether H_2S directly affects CHOP expression. One possible target of H_2S was demonstrated in a study by Lefer and colleagues (33), who found that, in myocardial ischemic injury, H_2S treatment provides protection in the heart through Nrf2 signaling, a master regulator of cytoprotective genes. Furthermore, mouse embryonic fibroblasts deficient in Nrf2 display augmented CHOP expression after glucose deprivation (60). Thus, we hypothesized that H_2S could inhibit LPS-induced CHOP by increasing Nrf2 signaling. Our results show that H_2S increases Nrf2 levels in macrophages and that silencing of Nrf2 abolishes the inhibitory effect of H_2S on LPS-induced CHOP upregulation. A molecular mechanism by which H_2S modulates Nrf2 was recently described. Under basal conditions, Nrf2 is maintained in the cytoplasm by binding to its negative regulator Keap1. H_2S S-sulfhydrates Keap1 at specific cysteine residues, resulting in dissociation of Keap1 from Nrf2 and enhanced Nrf2 nuclear translocation and transcriptional activity (61).

How does CHOP regulate the inflammatory response? CHOP is an atypical transcription factor because it can inhibit or enhance transcription of a target gene, depending on the interaction with other transcription factors (62, 63). A very recent study (11) demonstrated that CHOP can modulate NF-κB activation by decreasing IκB degradation and p65 translocation. This mechanism leads to a decrease in NF-κB target genes involved in apoptosis and inflammation. Thus, modulation of CHOP by H_2S can interfere with different pathways involved in the immune response during sepsis.

In summary, we report that H_2S treatment increased survival in a model of experimental sepsis by reducing the inflammatory response and the immunosuppressive state induced by apoptosis of immune cells, leading to a more effective and balanced innate and acquired immune response. Our data also highlight a major role for CHOP, which may act as an amplifier of the inflammatory response in the pathogenesis of sepsis, and the ability of H_2S treatment to counter CHOP signaling. Finally, H_2S inhibits CHOP expression, at least in part, through a mechanism involving Nrf2 activation. Sepsis is a complex and dynamic syndrome. These results increase our knowledge of the pathogenetic mechanisms of sepsis and simultaneously shed light on new targets and suggest innovative strategies for the treatment of this syndrome.

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Disclosures

The authors have no financial conflicts of interest.

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Corrections

Ferlito, M., Q. Wang, W. B. Fulton, P. M. Colombani, L. Marchionni, K. Fox-Talbot, N. Paolocci, and C. Steenbergen. 2014. H_2S increases survival during sepsis: protective effect of CHOP inhibition. *J. Immunol.* 192: 1806–1814.

In the title, the abbreviation "H_2S" should have been spelled out. The correct title is shown below.

Hydrogen Sulfide Increases Survival during Sepsis: Protective Effect of CHOP Inhibition

In addition, Fig. 1B, showing survival of mice treated with H_2S after induction of sepsis (H_2S posttreatment), was incorrect as printed. Fig. 1B was a duplicate of Fig. 1A. The correct Fig. 1B is printed below. The legend was correct as published and is shown below for reference.

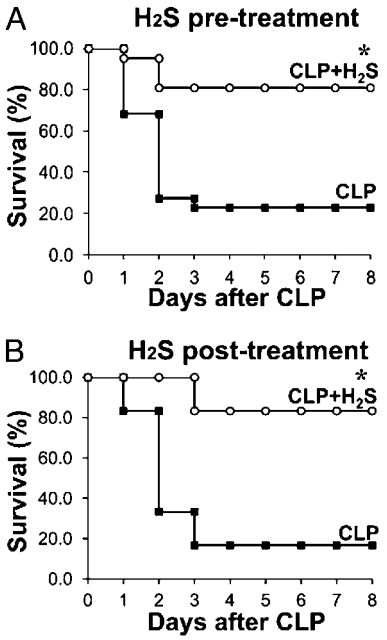

FIGURE 1. H_2S increases survival after sepsis. (**A**) Survival study of mice monitored for 8 d after CLP. A group of mice received NaHS (100 μmol/kg) as an H_2S donor s.c. (CLP+H_2S) or saline, and CLP was carried out after 1 h (CLP, $n = 21$; CLP+H_2S; $n = 22$). Data are pooled from three experiments. *$p = 0.0001$. (**B**) Sepsis was induced in mice by CLP, and H_2S was administered, as described above, 2 h later (both groups, $n = 6$). *$p = 0.01$.

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