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Delayed treatment with sodium hydrosulfide improves regional blood flow and alleviates cecal ligation and puncture (CLP) - induced septic shock

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Abstract

Cecal ligation and puncture (CLP) induced sepsis is a serious medical condition, caused by a severe systemic infection resulting in a systemic inflammatory response. Recent studies have suggested the therapeutic potential of donors of hydrogen sulfide (H₂S), a novel endogenous gasotransmitter and biological mediator in various diseases. The aim of the present study was to assess the effect of H₂S supplementation in sepsis, with special reference to its effect on the modulation of regional blood flow. We infused sodium hydrosulfide (NaHS), a compound that produces H₂S in aqueous solution (1, 3 or 10 mg/kg/h, for 1 hour at each dose level) in control rats or rats 24 hours after CLP, and measured blood flow using fluorescent microspheres. In normal control animals, NaHS induced a characteristic redistribution of blood flow, and reduced cardiac, hepatic and renal blood flow in a dose-dependent fashion. In contrast, in rats subjected to CLP, cardiac, hepatic and renal blood flow was significantly reduced; infusion of NaHS (1 mg/kg/h and 3 mg/kg/h) significantly increased organ blood flow. In other words, the effect of H₂S on regional blood flow is dependent on the status of the animals (i.e. a decrease in blood flow in normal controls, but an increase in blood flow in CLP). We have also evaluated the effect of delayed treatment with NaHS on organ dysfunction and the inflammatory response by treating the animals with NaHS (3 mg/kg) intraperitoneally (i.p) at 24 h after the start of the CLP procedure; plasma levels of various cytokines and tissue indicators of inflammatory cell infiltration and oxidative stress were measured 6 hours later. After 24 h of CLP, glomerular function was significantly impaired, as evidenced by markedly increased (over 4-fold over baseline) blood urea nitrogen and creatinine levels; this increase was also significantly reduced by treatment with NaHS. NaHS also attenuated the CLP-induced increases in malondialdehyde levels (an index of oxidative stress) in heart as well as in liver and myeloperoxidase levels (an index of neutrophil infiltration) in heart and lung. Plasma levels of IL-1 β , IL-5, IL-6, TNF- α and HMGB1 were attenuated by NaHS. Treatment of NaHS at 3 mg/kg i.p, (but not 1 mg/kg or 6 mg/kg), starting 24 hours post-CLP, with dosing repeated every 6 h, improved the survival rate in CLP animals. In summary, treatment with

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Competing interests

The authors declare no conflicts of interest in relationship to this study.

Authors' contributions

Akbar Ahmad: conduction of experiments, analysis of data, preparation of manuscript. Nadiya Druzhyna: conduction of experiments. Csaba Szabo: experimental design, preparation of manuscript.

3 mg/kg H₂S - when started in a delayed manner, when CLP-induced organ injury, inflammation and blood flow redistribution have already ensued - improves blood flow to several organs, protects against multiple organ failure, and reduces the plasma levels of multiple pro-inflammatory mediators. These findings support the view that H₂S donation may have therapeutic potential in sepsis.

Keywords

vascular tone; multiple organ failure; inflammation; therapeutic window; H₂S

Introduction

Hydrogen sulfide (H₂S), produced by three different enzymes, cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST) emerges as an endogenous gaseous biological mediator, with multiple regulatory roles in the nervous system, cardiovascular system and the immune system (1–7). Among others, H₂S regulates vascular tone, mitochondrial function and inflammatory signal transduction (1–7).

Both the donation of H₂S and the pharmacological inhibition of H₂S synthesis have been demonstrated to exert beneficial or detrimental effects in various experimental models of critical illness (reviewed in 7,8). While these effects have been placed in the context of modulation by H₂S of cell injury, inflammatory mediator production and mitochondrial function/cellular metabolism, less emphasis has been put on the potential effect of H₂S on the modulation of regional blood flow in critical illness. The goal of the current study was, therefore, in a rat model, to compare the effect of H₂S on regional blood flow under normal (physiological) conditions with its effect in a model of sepsis induced by cecal ligation and puncture (CLP), when applied in a post-treatment regimen. Since the data demonstrated that post-treatment with H₂S exerts beneficial effects on the blood flow of several parenchymal organs, we have also evaluated the effect of H₂S post-treatment on organ function, inflammatory response and survival.

Materials & Methods

Materials

Unless stated otherwise, all chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

In vivo studies

All investigations confirm to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (8th Edition, 2011), and was approved by the local ethics committee.

Animals and experimental design

Male Sprague Dawley rats (250–300 g) were housed in a light-controlled room with a 12 h light–dark cycle and were allowed ad libitum access to food and water. Animal care and

experimental protocols were performed in accordance with the IACUC, University of Texas Medical Branch, Galveston, TX, USA.

Regional blood flow measurements

Animals were anesthetized with isoflurane throughout the experiment. Operative sites were prepared with 10% povidone iodine solution. The right carotid artery, and the right femoral artery were cannulated with polyethylene tubing (PE 50) prefilled with heparinized saline solution. Mean arterial blood pressure (MAP) and heart rate (HR) were continuously monitored. The tubing in the right carotid artery was further advanced into the left ventricle, as confirmed by the typical left ventricle pressure curve (approximately 100 mmHg); left ventricular pressure was continuously monitored. The left femoral vein was cannulated for drug or vehicle infusion.

Fluorescent polystyrene microspheres containing green fluorescent dyes (absorption/emission wavelength 450/480 nm), carmine (580/620 nm) and yellow (515/534 nm) were used. Microspheres were vortexed for one minute, followed by sonication for one minute, to prevent flocculation. After sonication, 0.3 ml of the microsphere solution, approximately 300,000 microspheres, was aspirated into a 1 ml syringe. The right femoral artery catheter and the right carotid artery catheter were temporally disconnected from the monitor before injection. The carotid artery catheter was connected to the 1 ml syringe containing the microsphere solution of a chosen color. The right femoral artery catheter was connected to a peristaltic roller pump preset to remove blood at a rate of 1.5 ml/min into a test tube. Twelve seconds after the beginning of the removal of blood, 0.3 ml of the microsphere solution was injected into the carotid artery catheter over 20 seconds. Blood removal lasted for a total of 90 seconds. The carotid artery catheter was flushed with 2 ml of lactated Ringers solution during the last 60 seconds of blood removal to prevent microspheres adhesion to the inner surface of the catheter and to replace the volume of blood removed. The above procedure was subsequently repeated fluorescent microspheres of a different color (9).

At the end of each of the above experiments, the heart, lungs, liver, the right and the left kidneys were removed and individually weighed. All organs were individually placed inside a centrifuge tube (35 mm × 105 mm) with tissue digestion solution (2M KOH 44.8g + Tween 80 (0.5%) 8ml + 99% IMS ethanol) for 6 h in a water bath. Tubes were then vortexed and sonicated until complete dissolution of the fragments, followed by centrifugation (1500 g for 15 minutes). The supernatant was carefully aspirated leaving the pellet with microspheres. To prevent microspheres flocculation, 1 ml of dH₂O was added to the pellet, which was briefly vortexed, followed by the addition of 9 ml of ethanol-Tween (100% ethanol + 0.5% Tween-80). The resuspended pellet of microspheres was centrifuged at 1500 g for 15 minutes, the supernatant carefully aspirated, 5 ml of 0.1 M phosphate buffer (pH 7.0), and 4 ml of absolute alcohol were added to the pellet and vortexed briefly before spinning at 1500 g for 20 minutes. The supernatant was carefully aspirated and 4 ml of ethyl acetate-98% were added to the pellet and vortexed several times over 3–5 minutes. Tubes were kept in a dark room for 10 minutes to avoid photo bleaching of the fluorescent dyes, and readings were performed within one hour with a spectrofluorometer preset to determine

fluorescence within excitation and emission wavelengths of each color of microsphere used in the study (18).

After measuring the wavelength of all individual samples, blood flow was calculated from the measurement of fluorescence intensity for tissue and reference blood samples as follows:

$$Q = (At/Ab) \times (s/w) \times 100$$

Where Q is blood flow (in ml/min/100 g), At is individual sample intensity, Ab is reference blood sample intensity, s is reference blood sample withdrawal speed (ml/min), and w is tissue weight (g).

Blood flow measurements were conducted in (a) normal control animals (to obtain basal values); (b) in normal control animals subjected to sequential intravenous (i.v.) infusions of a freshly prepared solution of NaHS at a dose of 1, 3, or 10 mg/kg/h (1 h duration for each dose level), and (c) in animals after 24 h of CLP under baseline conditions, and (d) in animals subjected to CLP (protocol as below) for 24 h, followed by anesthesia, cannulation and sequential i.v. NaHS infusions of 1, 3, or 10 mg/kg/h (1 h duration of the infusion for each dose level).

Cecal ligation and puncture (CLP)

Sepsis was induced in rats by cecal ligation and puncture (CLP) as previously described (10). Briefly, rats were anesthetized with isoflurane throughout the experiment. The abdomen was shaved, wiped with 70% isopropanol and a midline abdominal incision (1 to 2 cm) was performed. The cecum was exteriorized, ligated with a sterile silk suture 1 cm from the tip and double punctured with a 20-gauge needle. The cecum was squeezed to assure expression of a small amount of fecal material and was returned to the abdominal cavity. The incision was closed with auto-clips and kept clean by povidone-iodine (Betadine). Rats were resuscitated with i.p. injection of 1 ml of lactated Ringer's solution. Sham-operated rats were treated as described above with the exception for the ligation and puncture of the cecum. Pain was prevented by buprenorphine (0.1 mg/kg; subcutaneous s.c. 30 minutes before surgery and every 12 hours thereafter).

At 24 hours of CLP, animals were either anesthetized and cannulated and subjected to blood flow measurements in response to i.v. infusions of NaHS (as described above), or animals were not anesthetized, but subjected to i.p. doses of vehicle or NaHS, followed by either survival studies (where animals were monitored until 48 hours post-CLP), or biochemical studies where the animals were killed at 30 hours post-CLP, and organs and plasma samples were collected and biochemical and inflammatory parameters and indices of organ injury were measured (as described below).

Experimental groups

For the first set of experiments, normal control Sprague Dawley rats (without CLP) were anesthetized and randomly allocated into the following groups: Sham (vehicle i.v. infusion); NaHS (1 mg/kg/h i.v.) infusion for 1 hour, followed by NaHS (3 mg/kg/h i.v.) infusion for 1 hour, followed by NaHS (10 mg/kg/h i.v.) infusion for 1 hour. The exact experimental

procedure for this study is described above, in the section "*Regional blood flow measurements*".

For the second set of experiments, Sprague Dawley rats were subjected to CLP (as described above), then monitored for 24 hours, then anesthetized (as above) and randomly allocated into the following groups: Sham group (vehicle infusion for 1 hour) or NaHS (1 mg/kg/h i.v.) infusion for 1 hour, followed by NaHS (3 mg/kg/h i.v.) infusion for 1 hour. In the initial studies, the original plans for the above protocol called for a third, higher dose of NaHS (10 mg/kg/h i.v.) infusion as well; however, after we have noted that animals were unable to tolerate this latter dose of NaHS, the highest dose was abandoned and the experiments only were conducted with 1 and 3 mg/kg/h i.v. NaHS doses. The exact experimental procedure for this study is described above, in the section "*Regional blood flow measurements*".

In the third set of experiments, rats were subjected to CLP for 24 hours, and then (without anesthesia) animals were randomized to either vehicle or NaHS treatment (in three separate groups of animals 1 mg/kg or 3 mg/kg or 6 mg/kg, i.p. administration, which was repeated every 6 hours). Survival rates were monitored for 24 hours subsequent to the first injection of NaHS or vehicle, i.e. 48 hours after the initiation of the CLP procedure.

The fourth set of experiments was designed to study the effect of delayed NaHS treatment on plasma cytokine levels and various indices of organ injury. In this set of experiments, the first subgroup of rats was subjected to CLP and plasma and tissues were harvested at 24 hours. The second subgroup of rats was subjected to CLP for 24 hours, and then randomized to either vehicle treatment or NaHS treatment (3 mg/kg i.p.: one single dose). These animals remained conscious until they were sacrificed: 6 hours after the injection of NaHS or vehicle (i.e. 30 hours after the start of the CLP procedure). Plasma samples or organs were collected. Major organs were snap frozen and kept at -80°C until use.

Determination of tissue lipid peroxidation via the malondialdehyde assay

Tissue malondialdehyde (MDA) levels, an index of tissue oxidative stress, were detected in organ homogenates using a fluorimetric MDA-specific lipid peroxidation assay kit (Enzo Life Sciences, Farmingdale, NY) as described (11). The assay is based on the BML-AK171 method in which two molecules of the chromogenic reagent N-methyl-2-phenylindole (NMPI) react with one molecule of MDA at 45°C to yield a stable carbocyanine dye with a maximum absorption at 586 nm.

Myeloperoxidase activity assay

Myeloperoxidase activity was measured in organ homogenates using a commercially available myeloperoxidase (MPO) fluorimetric detection kit (Enzo Life Sciences) as described (11). The assay utilizes a non-fluorescent detection reagent, which is oxidized in the presence of hydrogen peroxide and MPO to produce its fluorescent analog. The fluorescence is measured at excitation wavelength of 530 to 571 nm and emission wavelength of 590 to 600 nm.

Measurement of biochemical parameters by the Vetscan analyzer

Blood samples (100 µl) were analyzed by using Vetscan analyzer for biochemical parameters (albumin, alanine aminotransferase, amylase, total bilirubin, blood urea nitrogen, calcium, phosphorus, creatinine, glucose, sodium, potassium, total protein, globulin) within 1 h of collection as described (12). Increases in blood concentrations of ALT were considered evidence of hepatic damage; increases in amylase levels were considered as signs of acute pancreatitis; increases in blood urea nitrogen (BUN) and creatinine were considered signs of impaired glomerular function.

Detection of plasma cytokine levels

Blood from all groups were collected in K₂EDTA blood collection tubes and centrifuged at 4 °C for 15 min at 2,000g within 30 min of collection. Plasma was isolated, aliquoted and stored at -80 °C until use. The EMD Millipore's MILLIPLEX™ MAP Mouse cytokine Magnetic Bead Panel 1 kit was used for the simultaneous quantification of the following analytes: IL-1α, IL-1β, IL-2, IL-5, IL-6, IL-10, IL-13, IL-18, INF-γ and TNF-α (Merck Millipore, Darmstadt, Germany). Luminex method (12) was used. The method uses a proprietary technique to internally color code microspheres with two fluorescent dyes and to create distinctly colored bead sets of 500 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres, each of which is coated with a specific capture antibody. After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with streptavidin-phycoerythrin (PE) conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere. The Luminex instrument acquires and analyzes data using the LuminexxMAP fluorescent detection method and the LuminexxPONENT™ acquisition software (Thermo Fisher Scientific, Waltham, MA, USA). HMGB1 plasma levels were measured by an ELISA kit specific for rat HMGB1 (ABIN416082) according to the manufacturers' instructions.

Measurement of changes in the expression of CBS, CSE, 3-MST in various tissues after CLP

Heart, lung, liver and kidney samples from sham controls or from animals subjected to CLP for 24 h were placed in RIPA buffer and sonicated (3 times for 10 seconds each). The supernatants were preserved and the protein concentration was determined by bicinchoninic acid (BCA) assay. Protein expression was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The supernatant extracts (40 µg/µl) were boiled in equal volumes of loading buffer (150 mM Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 15% β-mercaptoethanol; and 0.01% bromophenol blue) and were electrophoresed on 8–12% polyacrylamide gels. Following electrophoretic separation, the proteins were transferred onto PVDF membranes for western blotting. The membranes were blocked with Starting Block T20 (PBS) Blocking Buffer (Thermo Scientific, Waltham, MA) for 1 h. The following primary antibodies were used: CBS (GenTex), CSE (Proteintech), and 3-MST (Sigma Aldrich) and HRP-conjugated β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The primary antibodies were incubated overnight at 4°C and the membranes were washed twice in TBST. A secondary horseradish peroxidase-conjugated antibody (anti-rabbit; anti-mouse; Cell Signaling Technology, Danvers, MA) was

then applied at a dilution of 1:5,000 for 1 h. Over a 30-min period, the blots were washed twice in TBST, after which they were incubated in enhanced chemiluminescence reagents (SuperSignal Detection kit; Pierce, Rockford, IL). The band intensity of the original blots was quantified using GeneTools (Syngene; Synoptics Ltd., Cambridge, MA), normalized to β -actin expression.

Measurement of plasma H₂S levels after CLP

The concentration of H₂S in plasma was quantified with the H₂S-specific fluorescent probe 7-azido-4-methylcoumarine (AzMC) as described (13). Whole blood was collected 30 min later in K₂EDTA blood collection tubes and centrifuged at 4 °C for 15 min at 2000g. Plasma was isolated and H₂S levels were measured with AzMc. After incubation of 200 μ l of plasma with 10 μ M AzMc, the fluorescence was measured using SpectraMax M2 microplate reader at ex = 365, em = 450 nm. H₂S levels were calculated against a NaHS standard curve.

Statistical analysis

All values described in the text and figures are expressed as means $\pm$ SEM for n observations. Student's t-test, one-way ANOVA with Tukey's post hoc test were used to detect differences between groups; Prism version 5 for Windows (GraphPad Software). $p < 0.05$, $p < 0.01$ were considered statistically significant. Survival analyses were performed using the Kaplan-Meier test followed by a log-rank test (Chi square).

Results

Modulation of regional blood flow by NaHS under baseline conditions

The infusion of hydrogen sulfide donor (NaHS 1 mg/kg/h, 3 mg/kg/h and 10 mg/kg/h) in healthy rats induced a dose-dependent reduction in the cardiac blood flow (Fig. 1A) and renal blood flow (Fig. 1D). Hepatic blood flow was already substantially reduced by the lowest dose of NaHS, and it was not affected by the subsequent doses.

Modulation of regional blood flow by NaHS during CLP

After 24 h of CLP, cardiac, hepatic and renal blood flow showed marked decreases compared to healthy animals (Fig. 2). Infusion of NaHS (1 mg/kg/h and 3 mg/kg/h) dose-dependently enhanced these blood flow values. At 3 mg/kg, the blood flow values were restored to levels comparable to the values measured in healthy control animals (Fig. 2). However - and in stark contrast to healthy control animals, which tolerated the highest dose of NaHS infusion - at 10 mg/kg/h, NaHS induced a 100% mortality prior to the originally planned end of the 1 hour infusion.

Effect of NaHS on survival rate after CLP

In a separate set of studies, CLP induced sepsis was performed, and 24 hours later NaHS was administered at various dose levels (1, 3 or 6 mg/kg); the same dose was repeated every 6 hours later. Treatment at the 3 mg/kg NaHS dose level (cumulative dose: 12 mg/kg, administered over a 24-hour period) significantly improved the survival rate in CLP induced sepsis at 42–48 h post-CLP (Fig. 3b). However, reducing the dose of NaHS to 1 mg/kg i.p.

(cumulative dose: 4 mg/kg, administered over a 24-hour period), administered every 6 hours, no longer improved survival significantly, although a rightward trend was still noted (Fig. 3a). Increasing the dose to 6 mg/kg i.p. every 6 hours (cumulative dose: 24 mg/kg, administered over a 24-hour period) did not affect CLP-induced mortality (Fig. 3c).

Effect of NaHS on tissue malondialdehyde levels after CLP

Malondialdehyde (MDA) significantly increased in heart and liver samples in response to CLP by 24 h, while MDA levels in the lung and kidney remained unaffected. Between the 24th and the 30th hours post-CLP, there was a marked additional increase in MDA levels in the heart, while in the liver MDA levels at 24 hours and 30 hours post-CLP were comparable (Fig. 4A, C). Treatment with NaHS (3 mg/kg. i.p) at 24 h post-CLP substantially attenuated the rate of increase of MDA levels over the subsequent 6 hours in the heart, and reduced MDA levels in the liver (Fig. 4A, C).

Effect of NaHS on neutrophil infiltration after CLP

Myeloperoxidase (MPO) significantly increased in heart and lung samples in response to CLP by 24 h, while MPO levels in the liver and kidney remained unaffected. Between the 24th and the 30th hours, there no significant change in MPO levels in any of the vehicle-treated CLP animals, while in the animals that received NaHS at 24 hours, by the end of the subsequent 6 hours (i.e. by 30 hours post-CLP) significant decreases in heart and lung MPO levels were detected (Fig. 5A, B).

Effect of NaHS on biochemical indices of organ injury after CLP

CLP significantly increased ALT, amylase, blood urea nitrogen and creatinine levels by 24 hours, and for the subsequent 6 hours, most parameters did not show a marked further change, except BUN, which decreased by approximately 50% (Table 1). NaHS treatment (3 mg/kg., i.p.), applied at 24 hours post-CLP, significantly increased the rate of BUN decline (by 30 hours, BUN values were comparable to baseline controls), and decreased alanine aminotransferase and amylase levels (Table 1), indicating a beneficial effect of delayed H₂S therapy on hepatic, pancreatic and renal dysfunction post-CLP.

Effect of NaHS on plasma cytokine levels after CLP

Plasma levels of IL-1 α , IL-1 β , IL-2, IL-5, IL-6, IL-10, IL-13, IL-18 and TNF- α and were increased in septic shock animals by 30 hours post-CLP; in the animals that received NaHS (3 mg/kg) at 24 hours, by 30 hours IL-1 β , IL-5, IL-6 and TNF- α were lower than in the corresponding vehicle control group (Table 2). Moreover, delayed NaHS treatment (3 mg/kg i.p., given 24 h after the start of CLP) reduced plasma levels of HMGB1, when measured 6 hours later (i.e. 30 hours after the start of CLP) (Fig. 6).

Effect of CLP on the expression of CBS, CSE and 3-MST and on the plasma levels of H₂S

All three H₂S producing enzymes (CBS, CSE and 3-MST) were present in heart, lung, liver and kidney tissues. In most tissues, expression levels were unaffected by CLP, but CLP increased CBS expression in the liver and CSE expression in the heart samples by

approximately 40% (Fig. 7, Table 3). Plasma H₂S levels were unaffected by CLP at 24 hours (baseline control: 44±10 μM vs. CLP: 46±15 μM; n=5).

Discussion

The main findings of the current report can be summarized as follows: **(a)** The hemodynamic effect of H₂S administration to rats after CLP is the opposite to its effect in normal control animals. While in normal control animals NaHS causes a dose-dependent decrease in the flow of several organs, the same dose of NaHS cause an improvement (increase, reversal) of blood flow after CLP. **(b)** CLP induces tissue-dependent alterations in oxidative stress (MDA) levels and neutrophil infiltration (MPO levels); by 24 hours most of these changes have plateaued out and do not further increase by a subsequent 6-hour period (with the notable exception of MDA levels in the heart, which continue to increase); delayed administration of NaHS (24h post-CLP) attenuates this increase, or, in other tissues, leads to a decrease in some of these parameters. **(c)** There are marked impairments in organ function, as evidenced by circulating markers of organ injury; some of these markers (most notably, BUN levels, a marker of renal dysfunction) are improved (i.e. their recovery is facilitated) by delayed treatment of the animals with NaHS. **(d)** A host of pro-inflammatory cytokines are increased post-CLP; plasma levels of some (but not all) of these mediators, including IL-1β, IL-5, IL-6, TNF-α and HMGB1 are reduced by delayed treatment of the animals with NaHS, while levels of several other cytokines (including the anti-inflammatory cytokine IL-10) are not affected by H₂S administration. **(e)** In most organs, the expression of H₂S-producing enzymes is not affected by CLP in our current model (with the notable exceptions of CBS in liver and CSE in heart, which show an increase) and there is no detectable net change in circulating (plasma) H₂S levels in the current model, at least at the 24 hour post-CLP time point. **(f)** NaHS administration (3 mg/kg every 6 hours, i.e. a total dose of 12 mg/kg, administered over 24 hours), starting in a substantially delayed therapeutic regimen, improves survival rate after CLP. However, the therapeutic window of NaHS appears to be very small, a 3-fold decrease in the dose (1 mg/kg every 6 hours, i.e. a total dose of 4 mg/kg, administered over 24 hours), or a 2-fold increase in the dose (6 mg/kg every 6 hours, i.e. a total dose of 24 mg/kg, administered over 24 hours) does not affect mortality. Moreover, a 1-hour infusion of NaHS at a dose of 10 mg/kg - while well tolerated in healthy control animals - results in a marked increase in mortality in 24-hour post-CLP animals. Taken together, H₂S affects multiple parameters of inflammation, oxidative stress and organ injury in an organ-specific manner. Delayed administration of NaHS acts as an enhancer/facilitator of the resolving of the organ injury/systemic inflammatory response post-CLP; this conclusion is in line with other emerging data in models of inflammation implicating H₂S as facilitator of the resolution of inflammation (14). We hypothesize that some of the effects of NaHS shown in the current report may be related to its beneficial hemodynamic effects, while other components of its beneficial effect may be related to various other pharmacological and biological actions of H₂S, including effects on oxidative stress, inflammatory signal transduction, vascular tone, and cell death effector pathways. Some of the specific molecular mechanisms affected by H₂S are overviewed in (1–8).

Several prior studies were conducted, in order to clarify the various pathophysiological roles of H₂S in various forms of circulatory shock (endotoxic shock, septic shock), both in rodent

models and in large animal models (overviewed in 7,8,15). In the current paper, we will focus our discussions on prior studies employing the CLP model of sepsis. Almost a decade ago, a study by Zhang and colleagues conducted several series of experiments in CLP-induced sepsis in mice, and reported a significant increase in H₂S plasma levels, which was associated with an increased CSE gene expression and enzymatic activity in the liver (16,17). There were increases in MPO levels in the lung and liver post-CLP, a response, which was further exacerbated by a high dose of NaHS (10 mg/kg); the effect of lower doses of NaHS was not reported in their paper. Likewise, NaHS at 10 mg/kg increased the circulating levels of pro-inflammatory chemokines and cytokines (16,17). These findings are in line with our current results showing that 10 mg/kg NaHS is poorly tolerated post-CLP, but they are not necessarily in contrast with our findings showing that NaHS at lower doses exerts beneficial effects in CLP. The bell-shaped pharmacological properties of H₂S are well known; at lower concentrations, NaHS exerts many protective, anti-inflammatory and antioxidant actions, while at higher doses it causes pro-oxidant, pro-inflammatory and cytotoxic responses (1–6). The increase in circulating H₂S was noted by Zhang and colleagues at an earlier time point (8 hours) post-CLP, as compared to the time point selected for the measurement of plasma H₂S levels in our study (24 hours); it is conceivable that the changes in H₂S homeostasis post-CLP are time-dependent and tissue-dependent (as well as possibly species dependent).

In contrast to the above-discussed studies from the Bathia group, Cunha's group (18) and Ferlito and co-workers (19) have concluded that in murine models of CLP-induced sepsis, NaHS administration exerts beneficial effects. In the study of Cunha, circulating leukocyte CSE activity was found to increase after CLP (18). In addition, treatment of mice subjected to severe CLP with various doses of NaHS (30, or 100 µmol/kg, subcutaneously, which converts to 1.68 and 5.6 mg/kg doses, respectively) improved the survival rate of the animals, while a lower dose (0.56 mg/kg) had no effect (18). In the study of Ferlito, only the 100 µmol/kg dose (i.e. 5.6 mg/kg) was used, which extended survival, both as a pretreatment and as a 2-hour post-treatment (19). The effective doses used in the above-mentioned studies are comparable to the dose of 3 mg/kg used by our current study. Therefore, the results of our studies (benefit of H₂S administration in a rodent model of CLP) are in line with the findings of both Cunha and Ferlito. Ferlito's study also measured cytokine levels post-CLP, and found that NaHS decreased plasma TNF-α and IL-10 levels (19); this is in line with our findings showing that NaHS decreased plasma TNF-α levels. Moreover - and similar to the findings of Xu and colleagues (20), in our present study, NaHS was found to decrease HMGB1 plasma levels post-CLP. Given the fact that HMGB1 is a late-acting mediator of critical illness - its neutralization, even in later stages of the disease, affords significant therapeutic benefit (21) - it is conceivable that part of the mode of the delayed beneficial action of NaHS in the current study is related to its ability to decrease HMGB1 levels. It must also be pointed out that, H₂S (independently from its effect on HMGB1 on net circulating levels of HMGB1) may also directly react with HMGB1, affecting the redox state of its cysteine residues, and, consequently, modifying its biological activity (22,23). This may be an additional mode of beneficial action of H₂S in CLP, which, however, remains to be investigated in further studies.

Similar to Ferlito's findings (19), we have also observed that NaHS decreases tissue MPO levels in several organs (including the lung). However, in our experiments - in contrast to the findings of Ferlito and colleagues - plasma levels of the anti-inflammatory cytokine IL-10 were unaffected by NaHS. Please note, however, that in our study the administration of NaHS was substantially delayed (24 hours) relative to the start of CLP compared to the design employed by Ferlito and colleagues, which may account for the differential effect on IL-10 production.

What, then, are the mechanisms responsible for the beneficial effects of (low-dose) NaHS therapy in CLP sepsis? As mentioned above, and discussed in multiple review articles (1–6), H₂S can affect biological functions through a multitude of mechanisms, including regulation of (a) vascular function; (b) inflammatory signal transduction, and subsequent effects on inflammatory cell activation and cell death (c) cellular bioenergetics, (d) oxidative stress/redox processes. Some of these processes occur through H₂S-induced modulation of proteins via posttranslational modification (sulfhydration), through direct redox interactions (e.g. electron donation, scavenging of various species), as well as through modulation of the opening/closing state of various membrane channels (e.g. K_{ATP} channels) (1–6). Prior studies, specifically focusing on individual inflammatory pathways affected by H₂S in murine models of CLP have implicated the modulation by H₂S of C/EBP homologous protein 10 (19), CXCR2, L-selectin, CD11b and G protein-coupled receptor kinase2 (18) in the beneficial effects of H₂S. Although in our study we did not seek to specifically delineate the pathogenetic contribution of any single inflammatory pathway, the findings of the current study are, in general, in line with some of these mechanisms. For instance, we have provided evidence in our model of CLP sepsis for the modulation of vascular function by H₂S through measurement of regional blood flow (Fig. 2); we have demonstrated that H₂S donation in CLP sepsis affects cytokine production (Table 2); and we have shown that H₂S donation affects oxidative stress burden (MDA levels) in various tissues (Fig. 4). Moreover, the effect of H₂S on tissue MPO levels (Fig. 5) is consistent with the modulation by H₂S of inflammatory cell activation, and the effect of H₂S on markers of end-organ injury (Table 1) is consistent with the modulation of H₂S of cell death during sepsis. How all of the above alterations influence each other in CLP remains to be elucidated. It is conceivable, for example, that H₂S-induced suppression of pro-inflammatory cytokine production inhibits the tissue infiltration of neutrophils, which, in turn, suppresses oxidative stress burden in the tissue. It is also possible that H₂S-induced improvements in tissue blood flow improve tissue metabolism, reduce inflammatory and oxidative damage, and reduce the degree of parenchymal cell dysfunction/cell death, thereby attenuating multiple organ dysfunction (Fig. 8). The above sequence of events may be a feasible explanation for the effect of NaHS on hepatic function in the current study: NaHS improves blood flow in this organ, it reduces hepatic MDA levels and improves hepatic function (assessed by plasma levels of the liver injury marker ALT). However, we did not detect any change in liver MPO content after CLP (i.e. we have no evidence for the involvement of infiltrating mononuclear cells, at least not at the 24–30 hours post-CLP time points). In other organs, the relative relationship of the various pathophysiological events seems less clear-cut. For instance, in the kidney, NaHS improves organ function (evidenced by decreased circulating BUN levels), and this is associated (and may be a consequence of) improved renal blood flow; however, no

significant evidence for any CLP-associated increase in renal MDA or MPO levels was noted. It appears that the pathophysiological changes in our model of CLP induced sepsis are organ-specific. It is also likely that H₂S affects these changes through effects on a complex array of pathways and mechanisms, rather than acting on a single pathway or cellular target.

The exact sequence of pathophysiological events as well as the relative contribution of the various modes of protective actions of H₂S to the observed net effects remain to be elucidated in further studies. The mechanism that explains the opposite hemodynamic effects of H₂S in normal animals (where it decreases parenchymal blood flow) and in animals subjected to CLP (where it increases/normalizes in parenchymal blood flow) also remains to be elucidated. It is also interesting (and currently unexplained) how H₂S exerts such differential effects on various inflammatory cytokines in CLP (i.e. why is it that the plasma levels of certain cytokines are suppressed by NaHS administration, while others are not, even though the signal transduction pathways involved in their regulation are often rather similar).

There are several mechanistic questions that remain to be addressed in future studies. First of all, did the NaHS infusion alter the progression of infection and was organ colonization affected by NaHS in the current study? Importantly, H₂S can affect bacterial virulence and the sensitivity of bacteria to antibiotic treatment, at least *in vitro* (24,25) and H₂S can also affect host immune function (26–28). It is, therefore, also conceivable that H₂S (produced endogenously during sepsis, and/or when administered in the form of NaHS) may also have affected bacterial replication, organ invasion and/or the sensitivity of the bacteria to elimination by the immune system. Although the time course of the NaHS response - i.e. fact that the beneficial effect of NaHS was rather rapid (it was administered at 24 hours, and changes in various parameters were already affected 6h later) - tends to speak against this possibility, the issue of the role of H₂S in bacterial virulence/growth during sepsis remain to be addressed in future studies. Moreover, H₂S is also known to impact macrophage function (26); future studies are needed to address whether the effect of H₂S on macrophage function may have contributed to the reduced inflammatory response and/or to the potentially altered course of bacterial infection. n responsible for the improvements in outcome. Another, related question is the following: what is the precise mechanism through which delayed H₂S administration (in the form of NaHS) protected the rats from sepsis death? The severity of organ dysfunction in the current model was rather modest, and it is unlikely that the animals died of multiple organ failure. Autonomic or neurological dysfunction, however, may be potential contributing factors. A fatal collapse of cardiovascular function, e.g. due to severe maldistribution of blood flow, and/or due to cardiac failure may also be a potential causative factor to CLP-induced death (and protection against these changes may be a potential mode of the beneficial effect of NaHS). In order to further substantiate this possibility, follow-up studies are needed to investigate the changes in cardiac blood flow and cardiac output in the current model. In addition, the reduction of inflammatory mediator production (especially the reduction in the plasma levels of the delayed inflammatory mediator HMGB1) may also have contributed to the beneficial effect of NaHS, although the data presented in the current study show only association, but not causality. Another follow-up study may focus on the changes in the cellular signaling

pathways by which H₂S actually reduced inflammation in CLP. In multiple prior studies, in various cell types, various H₂S donors have been shown to suppress specific signaling pathways like NF- κ B and MAP kinases (1–6); the most likely hypothesis is that H₂S simultaneously affects multiple signaling pathways, perhaps in a cell-, tissue- and time-dependent manner.

The translational implication of the current findings (as well as of the findings of prior studies investigating the role of H₂S in sepsis) remains to be explored. On a superficial level, the current findings (especially the improvement of survival, when NaHS was applied at a very late stage of sepsis, i.e. 24h after CLP, which, to our knowledge, is the longest delay in the administration of a H₂S donor, relative to the initiation of circulatory shock/sepsis in an animal model), as well as the positive findings with H₂S in murine models of CLP on survival, as well as the positive effects of NaHS on bacterial clearance and antibacterial defenses (18,19) would suggest that H₂S donation may be a translationally relevant therapeutic approach for sepsis. This conclusion may be further supported by several additional preclinical studies in various models of sepsis, endotoxemia and inflammation (27–31) as well as by clinical observations showing H₂S levels are elevated in various forms of critical illness (7,32). However, we must point out that the survival experiment was terminated 48 hours after the start of the CLP, so we do not know if the animals that were alive at 48 hours could be considered "permanent" survivors, or, alternatively, the NaHS treatment only delayed the onset of death. Moreover, without a significant amount of additional studies we must remain cautious in speculating for the potential translational implications of the current findings. For example, there is an intense debate in the field about the applicability (or lack thereof) of murine studies of critical illness to study human disease (33). Moreover, as shown in the current study 3 mg/kg i.p. NaHS, administered every 6 hours, starting 24 h post-CLP was beneficial, while reducing the dose 3-fold or increasing it 2-fold had no significant effect, while one hour of 10 mg/kg/h i.v. infusion was severely detrimental. These findings are in line with prior studies (18,19,28–31) concluding that the therapeutic window of NaHS is relatively small (although perhaps not substantially smaller than the therapeutic index of many other drugs used by critical care physicians). If the toxicity of H₂S is primarily determined by its peak concentration, then one way of extending its therapeutic index would be to use slow-release donors; another potential direction may be to employ H₂S donors that specifically target certain subcellular compartments (e.g. mitochondria); a further approach may involve multifunctional drugs where a H₂S donating component is combined with a scaffold which has its own independent pharmacological actions (34,35).

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Abbreviations

ALT	alanine aminotransferase
AzMC	7-azido-4-methylcoumarine

BUN	blood urea nitrogen
CBS	cystathionine- β -synthase
CLP	cecal ligation and puncture
CSE	cystathionine- γ -lyase
H₂S	hydrogen sulfide
IL	interleukin
NaHS	sodium hydrogen sulfide
MDA	malondialdehyde
MPO	myeloperoxidase
3-MST	3-mercaptopyruvate sulfurtransferase
TNF	tumor necrosis factor

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Fig. 1. Effect of NaHS on regional blood flow in healthy control rats

In anesthetized rats, NaHS (1 mg/kg/h, 3 mg/kg/h and 10 mg/kg/h) was infused for 1 hour each and then regional blood flow was measured in healthy rats. NaHS dose-dependently decreased cardiac blood flow (A), pulmonary blood flow (B), hepatic blood flow (C) and renal blood flow (D). Relative changes are shown as mean $\pm$ S.E.M. of three independent experiments; * $p < 0.05$, ** $p < 0.01$ vs. resting blood flow.

Fig. 2. Effect of NaHS on regional blood flow in rats subjected to 24 hours of CLP

After 24 h of CLP, rats were anesthetized and NaHS (1 mg/kg/h or 3 mg/kg/h) was infused for 1 hour each and then regional blood flow was measured. NaHS (3 mg/kg/h) infusion significantly increased cardiac blood flow (A), pulmonary blood flow (B), hepatic blood flow (C) and renal blood flow. Relative changes are shown as mean $\pm$ S.E.M. of three independent experiments; * $P < 0.05$, ** $P < 0.01$ vs. resting post-CLP blood flow.

Fig. 3. Effect of NaHS on survival rate after CLP

In conscious animals subjected to CLP, 24 hours after the initiation of CLP, vehicle or NaHS treatment (1 mg/kg, 3 mg/kg or 10 mg/kg i.p. bolus was started. The same i.p. dose was repeated every 6 hours. There was an improved survival rate by 42–48 hours in the medium dose group (3 mg/kg) (b) but NaHS did not affect survival in the low dose (1 mg/kg) group (a) or the high dose (1 mg/kg) group (c) (n = 8–10 animals per group). At 48 hours post-CLP, all animals were sacrificed and the experiment was terminated. Survival was analyzed by the Chi square method. * $p < 0.05$ CLP+NaHS (3 mg/kg) vs. CLP (+vehicle treatment).

Fig. 4. Effect of NaHS on tissue malondialdehyde (MDA) levels after CLP

Animals were subjected to CLP. At 24 hours after CLP, one group of animals was sacrificed and tissues were collected. Another group of animals was subjected to CLP and 24 hours later was randomized into a vehicle control group or a NaHS group (3 mg/kg i.p., single dose). Six hours later (i.e. 30 hours after the initiation of CLP), animals were killed and tissues were collected. MDA levels significantly increased in heart and liver samples by 24 hours CLP. Over the subsequent 6 hours MDA levels were unaffected in the liver, but further increased in the heart. In animals that received NaHS (3 mg/kg) 24 hours after CLP, heart and liver MDA levels were reduced over the subsequent 6 hours, as compared to the animals that received vehicle at 24 hours. There were no significant changes in MDA levels in lung and kidney samples. Each column represents mean $\pm$ S.E.M of $n=10$ animals per group.

* $P<0.05$, ** $P<0.01$ shows significant differences between the indicated groups.

Fig. 5. Effect of NaHS on tissue myeloperoxidase (MPO) levels after CLP

The experimental design was identical to as described in the legend of Fig. 4. MPO levels significantly increased in heart and lung samples by 24 hours after CLP; they remained elevated 6 hours later, but and were attenuated significantly by NaHS (3 mg/kg, administered at 24 hours post-CLP). There were no significant changes in MPO in liver and kidney samples. Each column represents mean $\pm$ S.E.M of n=10 animals per group.

*P<0.05, **P<0.01 shows significant differences between the indicated groups.

Fig. 6. Effect of NaHS on plasma HMGB1 levels after CLP

The experimental design was identical to as described in the legend of Figs. 4 and 5.

HMGB1 levels significantly increased in the plasma by 24 hours CLP. Over the subsequent 6 hours HMGB1 levels further increased. In animals that received NaHS (3 mg/kg) 24 hours after CLP, HMGB1 levels were reduced over the subsequent 6 hours, as compared to the CLP animals that received vehicle at 24 hours. Each column represents mean $\pm$ S.E.M of $n=10$ animals per group. $**P<0.01$ shows significant differences between the indicated groups.

Fig. 7. Expression of CBS, CSE and 3-MST after CLP

Representative western blots showing the expression of H₂S-producing enzymes (CBS, CSE and 3-MST) in heart, lung, liver and kidney samples in sham animals and at 24 hours post CLP.

Fig. 8. Potential mechanisms/pathways affected by delayed NaHS treatment in CLP

Table 1

Effect of NaHS on biochemical parameters after CLP.

	Albumin (g/dl)	Alanine aminotransferase (U/l)	Amylase (U/dl)	Total bilirubin (mg/dl)	Blood urea nitrogen (mg/dl)	Calcium (mg/dl)	Phosphorous (mg/dl)	Creatinine (mg/dl)	Glucose (mg/dl)	Sodium (mEq/l)	Potassium (mEq/l)	Total protein (g/dl)	Globulin (g/dl)
Sham	4± 0.1	54±4	854±48	0.3±0.1	17±2	9±0.2	7±0.3	0.4±0.1	212±8	139±0.7	4±0.2	5±0.1	2±0.1
CLP (24h)	3±0.4	99±25 **	1580±245 **	0.5±0.1	92±17 **	9±0.4	10±1 *	0.7±0.1 *	99±18	143±2	5±0.1	5±0.2	2±0.2
CLP (24h+6h)	2±0.2	71±9 **	1556±245 **	0.4±0.1	47±13 *	9±0.3	9±1	0.5±0.1	121±10	143±0.8	5±0.5	5±0.2	3±0.2
CLP+NaHS (24h+6h)	2±0.1	52±2 #	1105±153 #	0.4±0.1	25±4 #	9±0.2	8±1	0.4±0.1	102±19 #	143±0.6	4±0.1	5±0.2	3±0.1

Sepsis significantly increased ALT and amylase levels in CLP as compared to the sham controls. Impaired glomerular function was evidenced by a markedly increased (over 4-fold over sham control baseline) BUN and creatinine levels. Data are shown as mean±S.E.M. of 10 samples per group;

* p<0.05,

**

p<0.01 shows significant differences between sham vs. CLP (at 24 hours) and

p<0.05 shows significant differences between CLP (at 30 hours post CLP) vs. CLP+NaHS (24 hours of CLP, followed by NaHS administration, followed by sample collection 6 hours later).

Table 2

Effect of NaHS on plasma cytokine levels after CLP.

	IL-1 α	IL-1 β	IL-2	IL-5	IL-6	IL-10	IL-13	IL-18	TNF- α	IFN- γ
Sham	2 $\pm$ 0.5	30 $\pm$ 2	42 $\pm$ 4	101 $\pm$ 9	36 $\pm$ 10	1 $\pm$ 0.1	32 $\pm$ 0.1	39 $\pm$ 10	7 $\pm$ 1	2 $\pm$ 0.1
CLP (24h+6h)	8 $\pm$ 1	407 $\pm$ 7**	111 $\pm$ 4*	217 $\pm$ 6*	1956 $\pm$ 56*	4719 $\pm$ 44*	74 $\pm$ 13*	103 $\pm$ 8*	59 $\pm$ 18**	51 $\pm$ 7*
CLP + NaHS (24h+6h)	13 $\pm$ 2	220 $\pm$ 10#	72 $\pm$ 14#	129 $\pm$ 13#	1344 $\pm$ 23#	4678 $\pm$ 67	64 $\pm$ 16	135 $\pm$ 44	19 $\pm$ 7#	42 $\pm$ 4

Data are shown as mean $\pm$ S.E.M. of 10 samples per group;

* p<0.05,

** p<0.01

p<0.05 shows significant differences between sham vs. CLP (30 hours post CLP) and

CLP (30 hours post CLP) vs. CLP+NaHS (24 hours of CLP, followed by NaHS administration, followed by sample collection 6 hours later).

Table 3

Expression of CBS, CSE and 3-MST after CLP.

		Densitometry units (CLP/control $\times$ 100)
CBS	Heart	108 $\pm$ 19%
	Lung	102 $\pm$ 10%
	Liver	138 $\pm$ 10% *
	Kidney	101 $\pm$ 10%
CSE	Heart	144 $\pm$ 12% *
	Lung	121 $\pm$ 16%
	Liver	106 $\pm$ 16%
	Kidney	132 $\pm$ 20%
3-MST	Heart	100 $\pm$ 10%
	Lung	118 $\pm$ 10%
	Liver	119 $\pm$ 15%
	Kidney	118 $\pm$ 10%

Densitometric analysis of the expression of hydrogen sulfide producing enzymes (CBS, CSE and 3-MST) in heart, lung, liver and kidney samples. Data (mean $\pm$ S.E.M. of n=3 determinations per group) are shown as CLP densitometry units, as percent of corresponding sham control values.

* p<0.05 shows significantly higher expression of the indicated enzyme after CLP, when compared to the expression level in normal (sham operated) animals.