Hydrogen sulfide attenuates the inflammatory response in a mouse burn injury model

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Received March 11, 2013; Accepted July 5, 2013

DOI: 10.3892/mmr.2013.1610

Abstract. Hydrogen sulfide (H2S) is a naturally occurring gaseous transmitter, which is important in normal physiology and disease. In the present study, the involvement of H2S in the regulation of the immune response induced by burn injury was investigated in mice. Adult male C57BL/6 mice were subjected to burn injuries and treated with vehicle (0.9% sodium chloride, NaCl; 100 ml/kg body weight; subcutaneously, s.c.) or the H2S donor (sodium hydrosulfide, NaHS; 2 mg/kg body weight; s.c.). Compared with the controls, mice which received burn injuries exhibited a significant decrease in plasma H2S levels. Moreover, the levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-8 significantly increased, while IL-10 levels were decreased, compared with that of the controls in the plasma of mice subjected to burn injuries. Myeloperoxidase (MPO) activity in the liver tissue of injured mice was also markedly higher compared with that of the control group. However, the administration of NaHS significantly decreased the levels of TNF-α, IL-6 and IL-8 but increased the levels of IL-10 in the plasma of mice subjected to burn injuries. In addition, the MPO activity was decreased by NaHS. These results suggested that H2S regulates the inflammatory response induced by burn injury by modulating the levels of TNF-α, IL-6, IL-8 and IL-10. Thus, it was proposed that the administration of the H2S donor, NaHS, may be a useful therapy against the exaggerated immune response that is associated with burn injury.

Introduction

Burn injuries, which are characterized by heat-induced tissue coagulation at the time of injury, constitute a global public health problem (1). The systemic effects of burn injuries include the release of inflammatory cytokines produced by inflammatory cells and the vascular endothelium. These cytokines regulate lymphocyte function through the activation of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IFN-α and IFN-β, or the inhibition of IL-10 and TGF-β immune responses (2). Inflammation is important in pathogenesis, however, the imbalance between inflammatory and anti-inflammatory cytokines induced by burn injury aggravates the inflammatory response. This may result in the development of sepsis or systemic inflammatory response syndrome due to immune dysfunction, which increases the risk of mortality (3,4). Thus far, advances remain limited in the manipulation of the inflammatory response to treat burn injuries.

Previously, the third signaling gasotransmitter, hydrogen sulfide (H2S), was demonstrated to exhibit physiological and physiopathological roles in vivo and in vitro (5,6). An increasing number of studies suggest that H2S exerts protective effects against various stimuli-triggered injuries in numerous organs, including the heart, liver and kidneys (7,8). However, the importance of H2S in inflammation is only recently beginning to be elucidated, and the exact role of H2S in inflammation remains controversial, as pro-inflammatory and anti-inflammatory effects have been demonstrated (9). Certain studies have determined the pro-inflammatory effects of H2S. These studies showed that inflammation was correlated with increased levels of plasma H2S and tissue H2S synthesizing enzyme activity. In addition, the inhibition of H2S synthesis by DL-propargylglycine (PAG) treatments led to reduced inflammation (10-12). However, other studies have observed anti-inflammatory effects of H2S. Treatments with either H2S releasing non-steroidal anti-inflammatory drugs or H2S donors (sodium hydrosulfide, NaHS) showed anti-inflammatory activity in various models of inflamma-
tion (13-15). In addition, in lipopolysaccharide-stimulated microglias and astrocytes, \( \text{H}_2\text{S} \) exerted an anti-inflammatory effect (16).

In the present study, it was hypothesized that \( \text{H}_2\text{S} \) is important in the regulation of the inflammatory response induced by burn injury. Thus, the therapeutic potential of NaHS, an \( \text{H}_2\text{S} \) donor in an *in vivo* model of burn-related inflammation in mice, was investigated. It was determined that \( \text{H}_2\text{S} \) was anti-inflammatory and suppressed the inflammatory response associated with burn injury.

**Materials and methods**

**Animals.** Adult male C57BL/6 mice (age, 6-8 weeks; weight, 21-24 g) were obtained from the Second Military Medical University (Shanghai, China). Mice were individually housed in laminar flow cabinets under specific pathogen-free conditions with access to food and water ad libitum. The animals were acclimatized for 1 week prior to the experiment, and maintained throughout at standard conditions of 50% relative humidity, 24±1°C and a 12-h light-dark cycle. The mice were randomized into two groups; the first was a sham group that did not receive burn injuries (n=7) and the second group received burn injuries. Mice in the second group were then randomly subdivided into three groups (n=7 per group): An untreated group, a saline group that received the vehicle (0.9% sodium chloride, NaCl; 100 ml/kg body weight; subcutaneously, s.c.) and an NaHS group that was treated with NaHS (2 mg/kg body weight; s.c.). All animal experiments were conducted according to the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals (2011, ISBN-13: 978-0-309-15400-0), and were approved by the Institutional Animal Care and Use Committee of the Second Military Medical University.

**\( \text{H}_2\text{S} \).** The dehydrated NaHS powder (anhydrous; Beijing Chemical Reagents Company, Beijing, China) was dissolved in isotonic saline (0.9%) immediately prior to administration. For the treatment groups, either normal saline or NaHS were subcutaneously injected into the mice using a 32-gauge needle.

**Mouse injury model.** The burn injury model was generated as described previously (17). Briefly, mice were anesthetized by intraperitoneal injection of pentobarbital sodium (75 mg/kg), then the dorsum was cleansed and shaved with electrical hair clippers. Subsequently, 40% of the total body surface area (TBSA) was exposed to a 95°C water bath for 10 sec, followed by 4°C water for 45 sec to halt the burning process. A full-thickness skin burn was confirmed by its characteristics with the loss of epidermis and dermis. Mice were revived from unconsciousness by intraperitoneal application of 1 ml sterile saline 1 h after burning. The group of mice without exposure to boiling water served as controls. Mice were then immediately injected with the vehicle or sodium sulfide (2 mg/kg body weight, s.c.). After 10 h, mice were sacrificed by CO\(_2\) asphyxiation. Blood samples were taken via direct cardiac puncture for analysis by enzyme-linked immunosorbent assay (ELISA) assay. Liver samples from each group were collected and frozen immediately in liquid nitrogen for subsequent measurement of tissue myeloperoxidase (MPO) activity.

**Detection of plasma \( \text{H}_2\text{S} \) content.** Blood samples for each group were taken in heparinized tubes via direct cardiac puncture and centrifuged at 726 x g for 5 min. Plasma \( \text{H}_2\text{S} \) concentration was measured with an ELIT Ion Analyzer (ELIT 9801; Electro Analytical Instruments Ltd., London, UK) as described previously (18). In brief, 0.5 ml sulfide antioxidant buffer (SAOB; NaOH 2.35 mol/l and EDTA 0.27 mol/l) was added to 0.5 ml \( \text{H}_2\text{S} \) standard solutions (10, 20, 30, 40, 50, 60 and 80 µmol/l, respectively). A sulfide-sensitive electrode (ELIT 8225) and a reference electrode (ELIT 003n; Electro Analytic Instruments Ltd.) were rinsed in deionized water, blotted dry and immersed in a mixture of SAOB and 10 µmol/l \( \text{H}_2\text{S} \) standard solution. When a stable reading was displayed, the voltage value (mV) was recorded. This procedure was repeated for the other combinations of SAOB and different concentrations of \( \text{H}_2\text{S} \) standard solutions. When all the standards were measured, the standard curve of voltage versus concentration was plotted. The electrodes were washed as previously, and the samples were measured in the same way as the measurement for the standard solution. The sample data were plotted on the standard curve and the sample concentration was obtained.

**Measurement of MPO activity.** Neutrophil sequestration in the liver was quantified by measuring the tissue MPO activity as previously described (19). Liver samples were thawed, homogenized in 20 mM phosphate buffer (pH 7.4), centrifuged (10,000 x g for 10 min at 4°C) and the resulting pellet was resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% (w/v) hexadecyltrimethylammonium bromide (Sigma-Aldrich, St. Louis, MO, USA). The suspension was subjected to four cycles of freezing and thawing, and further disrupted by sonication (40 sec). The sample was then centrifuged (10,000 x g for 5 min at 4°C) and the supernatant was used for the MPO assay. The reaction mixture consisted of the supernatant (50 µl), 1.6 mM tetrathiomethane (Sigma-Aldrich), 80 mM sodium phosphate buffer (pH 5.4) and 0.3 mM hydrogen peroxide (50 µl). This mixture was incubated at 37°C for 110 sec, the reaction was terminated with 50 µl of 0.18 M H\(_2\)SO\(_4\), and the absorbance was measured at 450 nm. The absorbance was then corrected for the DNA content of the tissue sample and results were expressed as the enzyme activity.

**Measurement of plasma inflammatory and anti-inflammatory cytokine levels by ELISA.** Blood samples from each group were collected in heparinized tubes via direct cardiac puncture and centrifuged at 726 x g. Cytokine levels were quantified by a sandwich ELISA using ELISA kits (Quantikine® Colorimetric Sandwich ELISAs; R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Briefly, samples (100 µl) and IL-6 standards (0, 62.5, 125, 250, 500 and 1000 pg/ml) were added to the wells. Each was tested in duplicate. Following 1 h of incubation at 37°C, samples were removed and the plates were washed with a washing buffer (consisting of PBS, 10 mmol/l, pH 7.4 and Tween 20, 0.1%). Anti-rat IL-6 biotin (100 µl) was added to each well of the plates and left for 30 mins at 37°C. Following five additional washing steps, 100 µl horseradish peroxidase was added to the wells and left for 30 mins at 37°C. Subsequent to a further wash,
100 µl tetramethylbenzidine substrate was added to each well for color development. The mixture was incubated in the dark for 30 mins at room temperature. Following the termination of the reaction by the addition of 100 µl stop solution to each well, the optical density (OD) values at 450 nm were measured by a Bio-Rad ELISA reader (iMark Microplate Absorbance Reader; Bio-Rad, Richmond, CA, USA). The standard curve of the OD value versus the concentration of IL-6 was obtained. The sample data were plotted on the standard curve and the sample IL-6 concentration was determined. The same method was utilized for the analysis of the plasma levels of TNF-α, IL-8 and IL-10.

Statistical analysis. SPSS Version 17.0 software (SPSS for Windows, Inc., Chicago, IL, USA) was used for all statistical analyses. All results are presented as the mean ± SEM. Statistical analysis of the data was performed using standard one-way analysis of variance followed by a least significant difference post hoc test. Bonferroni's correction was used to adjust for multiple comparisons. A two-tailed Student's paired t-test was also used to compare the difference in values between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Burn injury decreases the H₂S levels in the plasma. As shown in Fig. 1, H₂S levels in the plasma of mice were significantly lower in the groups subjected to burn injury compared with those of the sham group (P<0.05). In addition, NaHS significantly increased the plasma H₂S levels compared with those of the burn control group that received no treatment (P<0.05).

Burn injury promotes the inflammatory response in rats, which is suppressed by the H₂S donor, NaHS. An ELISA assay demonstrated that the levels of IL-6, IL-8 and TNF-α in the plasma of mice subjected to burn injuries were significantly higher than those of the sham group that received no injury (P<0.01, Fig. 2). Notably, the plasma levels of IL-10 in the injured group were significantly decreased compared with those in the sham group (P<0.01, Fig. 2). Administration of NaHS in the mice that had received burn injuries significantly decreased the IL-6 levels in the plasma compared with those that received saline or no treatment (P<0.05, Fig. 2). Similarly, administration of NaHS significantly decreased the IL-8 and TNF-α levels in the plasma of injured mice compared with those that received saline or no treatment (P<0.05, Fig. 2). However, the H₂S donor treatment significantly enhanced the IL-10 levels in the plasma of injured mice compared with those that received saline or no treatment (P<0.05, Fig. 2). No significant differences were identified between the saline and the untreated groups.

NaHS decreases the enhanced MPO activity induced by burn injury. Tissue MPO activity, an indicator of neutrophil inflammatory activity, was markedly increased in the livers of mice subjected to burn injury compared with that of the sham group, indicating increased leukocyte infiltration in the mouse liver (Fig. 3, P<0.01). However, treatment with NaHS (2 mg/kg of body weight, s.c.) significantly reduced the MPO activity (Fig. 3, P<0.05).

Discussion

Burn injuries remain one of the most widespread and devastating forms of trauma and are ranked among the
leading causes of injury-related morbidity and mortality worldwide (20,21). Although inflammation is important in the pathogenesis of burn injuries, severe burns often lead to systemic inflammatory response syndrome (SIRS), which may be responsible for the majority of the associated morbidity and mortality (22,23). H2S may be important in the regulation of the inflammatory process, however, the effects of H2S on inflammation are controversial (10,24,25). In the present mouse model, a predominant anti-inflammatory effect of H2S was observed. Burn injuries resulted in a significant reduction in the H2S levels in the plasma and an exaggerated inflammatory response was also observed, which was identified by significantly increased levels of IL-6, IL-8 and TNF-α, and a decreased level of IL-10. The MPO activity in the liver tissue of injured mice was also markedly increased. However, administration of NaHS, an H2S donor, alleviated the immune response, as demonstrated by the upregulated levels of a plasma anti-inflammatory cytokine (IL-10) and the reduced generation of pro-inflammatory cytokines (IL-6, IL-8 and TNF-α). In this model, H2S also decreased the elevated MPO activity of the liver tissue induced by burn injury.

H2S has been demonstrated to be an essential mediator of severe burn injury-induced inflammation in mice (9). The results of the present study in mice showed that a 40% TBSA full thickness burn induced a significant decrease in plasma H2S levels. Inflammatory cytokines exert well-characterized effects on the pathogenesis of severe burn-induced injury (26). IL-6 is produced by numerous cell types, including monocytes/macrophages, endothelial cells, fibroblasts and smooth muscle cells, in response to stimulation by endotoxin, IL-1β and TNF-α (27,28). Circulating levels of IL-6 are strong predictors of the severity of SIRS. The importance of IL-6 in the acute-phase response has been demonstrated by its ability to stimulate the synthesis of acute-phase proteins, including C reactive protein, from hepatocytes in vitro and in vivo (29). Patients with systemic inflammatory conditions, such as sepsis or SIRS, also exhibit increased circulating levels of IL-8 (30). In acute pancreatitis, increased IL-8 levels predicted the severity of the disease (31). The anti-inflammatory cytokine IL-10 is a central immunosuppressive cytokine that regulates the innate and adaptive immune responses, resulting in inhibition of the alveolar macrophage production of pro-inflammatory mediators involved in SIRS (32). Increased IL-10 plasma levels in animal models of endotoxemia may inhibit the release of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α, from monocytes and macrophages, thereby preventing subsequent tissue damage (33). In the present study, it was determined that the levels of TNF-α, IL-6 and IL-8 in the plasma were significantly increased, while IL-10 secretion was inhibited by burn injury. Administration of NaHS significantly decreased the TNF-α, IL-6 and IL-8 levels in the plasma, but elevated the IL-10 plasma levels. Inflammatory cytokines are essential in systemic immune dysfunction. The results demonstrated that NaHS decreased the levels of inflammatory cytokines and increased those of the anti-inflammatory cytokines, which suggested that H2S may provide protection by alleviating the exaggerated inflammatory damage associated with burn injury. However, further studies are required to determine the precise mechanisms by which H2S regulates the inflammatory response.

As leukocyte recruitment is pivotal in the pathogenesis of organ injury caused by burn injury (19), it was investigated whether NaHS is able to protect against organ injury by reducing leukocyte recruitment. It was determined that burn injury significantly enhanced the MPO activity in the liver, indicating increased neutrophil infiltration in this organ. Moreover, NaHS treatment reduced the MPO activity in the livers of mice subjected to burn injury, indicating attenuated neutrophil infiltration. These results were in accordance with those of a study demonstrating that NaHS modulated leukocyte-mediated inflammation by decreasing leukocyte adhesion and leukocyte infiltration (34).

However, Zhang et al demonstrated that mice subjected to 30% TBSA burn injury (10) exhibited significantly elevated plasma and hepatic H2S levels, with a concomitant increase in liver and lung expression of cystathionine-β-synthase and cystathionine-γ-lyase (CSE), 8 h after injury. Prophylactic and therapeutic administration of PAG reduced burn-associated neutrophil accumulation and histological changes in the liver and lung tissues. Injection of NaSH (10 mg/kg; i.p.) at the same time as burn injury aggravated the burn-associated tissue damage and inflammation. These results are not concordant with those observed in the present study, and thus may be due to the different animal models and doses of H2S donors used. Severe full-thickness burn injury initially produces a large systemic inflammatory reaction characterized by leukocyte activation and plasma leakage in the microvasculature of tissues and organs remote from the wound. The degree of the inflammatory response correlates directly with the percentage of TBSA burnt (35). In our study, mice received a 40% TBSA burn injury, which was different from the previous animal model. Moreover, the dose and administration routes were different. In this study, the injected dose of NaHS was 2 mg/kg (s.c.), which was markedly lower than that in the study.
by Zhang et al (10 mg/kg; i.p.). It has been demonstrated that a high dose of H\textsubscript{2}S donor may aggravate inflammation and injury while a low dose of H\textsubscript{2}S decreases inflammation.

In conclusion, the present study demonstrated that in a murine model of inflammation induced by burn injury, administration of NaHS alleviated the immune response, as determined by the reversed MPO activity of the liver tissue, increased levels of an anti-inflammatory cytokine (IL-10) and reduced levels of pro-inflammatory cytokines (IL-6, IL-8 and TNF-\(\alpha\)). This study provides novel insights into the involvement of H\textsubscript{2}S in attenuating the burn-induced systemic immune response. Modulation of endogenous H\textsubscript{2}S or exogenous administration of H\textsubscript{2}S may be a novel therapeutic strategy for immune dysfunction induced by burn injury.

References