Hydrogen Sulfide and Neurogenic Inflammation in Polymicrobial Sepsis: Involvement of Substance P and ERK-NF-κB Signaling

Seah-Fang Ang¹,², Shabbir M. Moochhala³,⁴, Paul A. MacAry¹,², Madhav Bhatia⁵*

¹ Immunology Program and Department of Microbiology, Center for Life Sciences, National University of Singapore, Singapore, ² NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, Singapore, ³ Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, ⁴ Defense Medical and Environmental Research Institute, DSO National Laboratories, Singapore, ⁵ Department of Pathology, University of Otago, Christchurch, New Zealand

Abstract

Hydrogen sulfide (H₂S) has been shown to induce transient receptor potential vanilloid 1 (TRPV1)-mediated neurogenic inflammation in polymicrobial sepsis. However, endogenous neural factors that modulate this event and the molecular mechanism by which this occurs remain unclear. Therefore, this study tested the hypothesis that whether substance P (SP) is one important neural element that implicates in H₂S-induced neurogenic inflammation in sepsis in a TRPV1-dependent manner, and if so, whether H₂S regulates this response through activation of the extracellular signal-regulated kinase-nuclear factor-κB (ERK-NF-κB) pathway. Male Swiss mice were subjected to cecal ligation and puncture (CLP)-induced sepsis and treated with TRPV1 antagonist capsazepine 30 minutes before CLP. DL-propargylglycine (PAG), an inhibitor of H₂S formation, was administrated 1 hour before or 1 hour after sepsis, whereas sodium hydrosulfide (NaHS), an H₂S donor, was given at the same time as CLP. Capsazepine significantly attenuated H₂S-induced SP production, inflammatory cytokines, chemokines, and adhesion molecules levels, and protected against lung and liver dysfunction in sepsis. In the absence of H₂S, capsazepine caused no significant changes to the PAG-mediated attenuation of lung and plasma SP levels, sepsis-associated systemic inflammatory response and multiple organ dysfunction. In addition, capsazepine greatly inhibited phosphorylation of ERK1/2 and inhibitory κBα, concurrent with suppression of NF-κB activation even in the presence of NaHS. Furthermore, capsazepine had no effect on PAG-mediated abrogation of these levels in sepsis. Taken together, the present findings show that H₂S regulates TRPV1-mediated neurogenic inflammation in polymicrobial sepsis through enhancement of SP production and activation of the ERK-NF-κB pathway.

Introduction

The neuropeptide substance P (SP) is an 11 amino acid peptide encoded by the preprotachykinin-A (PPT-A) gene. It is distributed throughout the nervous system of human and animal species [1,2]. Belonging to the tachykinin family of neurotransmitters, SP is well recognized for its numerous potent neuroimmunomodulatory actions. Its biological activities are primarily mediated through neurokinin-1 receptor. SP has been established to exert a vast range of proinflammatory effects in vitro and in vivo, influencing many immune and inflammatory disorders of the respiratory, gastrointestinal, and musculoskeletal systems [2]. SP activates inflammatory cells to produce various inflammatory molecules such as cytokines, chemokines, reactive oxygen species, and arachidonic acid derivatives that potentiate tissue inflammation, and vasoactive substances like histamine and serotonin that promote vascular leakiness and edema at the injured tissue site [1,2]. Additionally, SP provokes lymphocyte proliferation, immunoglobulin production, leukocyte chemotaxis, and activation of proinflammatory transcription factors; all of which exacerbate tissue injury and amplify the overall inflammatory response [2]. Therefore, it is obvious that an extensive neuro-immune intersystem crosstalk exist between SP and the inflammatory response to injury.

Neurogenic inflammation encompasses a series of inflammatory responses triggered by the activation of primary sensory neurons and the subsequent release of inflammatory neuropeptides [3]. Of major importance in the development of neurogenic inflammation is the transient receptor potential vanilloid type 1 (TRPV1) receptor, a non-selective cation channel that is best characterized for its location on these neurons [4]. Besides integrating painful stimuli, TRPV1-expressing sensory nerve terminals play a dominant role in initiating neural inflammatory processes [3]. In particular, activation of TRPV1 by noxious stimuli, including hydrogen sulfide (H₂S), leads to depolarization with consequent release of neuropeptides such as SP that participates in neurogenic inflammation [5].

In recent years, H₂S has been recognized as the third endogenous signaling gasotransmitter, alongside carbon monoxide and nitric oxide. Cystathionine-β-synthase (CBS) in the central nervous system and cystathionine-γ-lyase (CSE) in the cardiovascular system are the

Citation: Ang S-F, Moochhala SM, MacAry PA, Bhatia M (2011) Hydrogen Sulfide and Neurogenic Inflammation in Polymicrobial Sepsis: Involvement of Substance P and ERK-NF-κB Signaling. PLoS ONE 6(9): e24535. doi:10.1371/journal.pone.0024535

Editor: Songtao Shi, University of Southern California, United States of America

Received: June 15, 2011; Accepted: August 11, 2011; Published: September 12, 2011

Copyright: © 2011 Ang et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by National Medical Research Council grants R-184-000-111-213 and R-184-000-156-213. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: madhav.bhatia@otago.ac.nz
Figure 1. Effect of NaHS and capsazepine on protein and mRNA levels of SP in septic mice. Mice were randomly given NaHS (10 mg/kg, i.p.) or vehicle (DMSO) at the same time of CLP; and capsazepine (Capz) (15 mg/kg, s.c.) or vehicle (DMSO) 30 minutes before CLP. Sham mice were used as controls. Eight hours after CLP or sham operation, (A) lung and (B) plasma SP levels, and (C) lung PPT-A mRNA levels were measured. Results shown are the mean values ± SEM (n = 8–12 mice per group). *P<0.01 versus sham; **P<0.01 versus CLP+vehicle; †P<0.05 versus CLP+vehicle; ‡P<0.01 versus CLP+NaHS+vehicle.
doi:10.1371/journal.pone.0024535.g001

Figure 2. Effect of NaHS and capsazepine on protein levels of cytokines and chemokines in the lungs and liver of septic mice. Mice were randomly given NaHS (10 mg/kg, i.p.) or vehicle (DMSO) at the same time of CLP; and capsazepine (Capz) (15 mg/kg, s.c.) or vehicle (DMSO) 30 minutes before CLP. Sham mice were used as controls. Eight hours after CLP or sham operation, protein levels of (A) TNF-α, (B) IL-1β, (C) IL-6, (D) MIP-1α and (E) MIP-2 were measured. Results shown are the mean values ± SEM (n = 8–12 mice per group). *P<0.01 versus sham; **P<0.01 versus CLP+vehicle; †P<0.05 versus CLP+vehicle; ‡P<0.01 versus CLP+NaHS+vehicle.
doi:10.1371/journal.pone.0024535.g002
key enzymes mostly responsible for the enzymatic production of H2S [6]. Besides acting as a vasodilator and neuromodulator [7,8], H2S functions as a cardiovascular modulator and was associated with vascular consequences of endotoxic and septic shock [9]. It also contributes to local and systemic inflammation seen in experimental models of hind paw edema, acute pancreatitis, endotoxemia, and cecal ligation and puncture (CLP)-induced sepsis [6]. Importantly, our earlier work has demonstrated that H2S promotes TRPV1-mediated neurogenic inflammation in polymicrobial sepsis [10]. However, endogenous neural factors that modulate this event have yet to be identified.

Sepsis is characterized by systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) [11]. Sepsis develops when the initial, appropriate host response to an infection becomes amplified, and then dysregulated. Microorganisms and their products implicate in aberrant inflammatory mediator production, such as cytokines and chemokines, which in turn upregulate adhesion molecules and stimulate leukocyte recruitment [11,12]. Activation of nuclear factor-κB (NF-κB) results in increased gene expression and biosynthesis of proinflammatory mediators in sepsis [13]. In addition, extracellular signal-regulated kinase (ERK) signaling has been shown to be important for the temporal control of NF-κB transcriptional activity and expression of NF-κB-regulated genes, as well as upstream activator of NF-κB [14,15]. Other studies also highlighted the significance of NF-κB and ERK in sepsis [14,15,16,17]. Furthermore, H2S was found to regulate inflammatory response in sepsis through activation of the ERK pathway [18]. However, none has investigated the detailed signaling mechanism of endogenous H2S in mediating neurogenic inflammation in polymicrobial sepsis [10].

Therefore, in the present study, we hypothesized that SP is one important neural element that implicates in H2S-induced neurogenic inflammation in sepsis through a TRPV1 channel-dependent mechanism, and that H2S regulates this response through activation of the ERK-NF-κB pathway.

Materials and Methods

Animal model of polymicrobial sepsis

All experiments were approved by the Animal Ethics Committee of National University of Singapore and were conducted in...
Figure 4. Effect of NaHS and capsazepine on liver dysfunction and lung edema in septic mice. Mice were randomly given NaHS (10 mg/kg, i.p.) or vehicle (DMSO) at the same time of CLP; and capsazepine (Capz) (15 mg/kg, s.c.) or vehicle (DMSO) 30 minutes before CLP. Sham mice were treated with 1 ml of saline s.c. Eight hours after CLP or sham operation, plasma levels of (A) ALT and (B) AST, and (C) lung wet-to-dry weight ratio were measured. Results shown are the mean values ± SEM (n = 10–15 mice per group). *P<0.01 versus sham; **P<0.01 versus CLP+vehicle; †P<0.05 versus CLP+vehicle. doi:10.1371/journal.pone.0024535.g004

Cytokines, chemokines, and adhesion molecules determination

Lung and liver samples were thawed, homogenized in 1 ml of ice-cold SP assay buffer for 20 seconds (Bachem, Peninsula Laboratories, San Carlos, CA, USA). The homogenates were centrifuged (10,000 g; 20 minutes, 4°C) and the supernatants collected. The supernatants and plasma were adsorbed on C18 cartridge columns (Bachem, Peninsula Laboratories) as previously described [35]. The adsorbed peptide was eluted with 1.5 ml of 75% (v/v) acetonitrile.

Measurement of pulmonary edema

As an index of lung edema, the amount of extravascular lung water was calculated according to established techniques [37,38,39]. Briefly, mice were killed 8 hours after surgery and blood was collected by cardiac puncture. The lungs were excised from mice, cleared of all extrapulmonary tissue, blotted and weighed (total lung wet weight); they were then dried in an incubator for 48 hours at 80°C and weighed again (total dry weight). For each animal, pulmonary edema was expressed as the ratio of total wet weight to total dry weight.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assay

Plasma ALT and AST activities were measured with the Infinity ALT and AST liquid stable reagent (Thermo Electron Corporation, Pittsburgh, PA, USA) according to the manufacturer’s instructions.

Nuclear extraction and measurement of NF-κB activation

Nuclear extracts from lung (50 mg) and liver (100 mg) were prepared by using a nuclear extraction kit as described by the manufacturer (Active Motif, Carlsbad, CA, USA). Protein concentrations in nuclear extracts were determined using a Bradford assay.
Figure 5. Effect of PAG and capsazepine on protein and mRNA levels of SP in septic mice. Mice were randomly given PAG (50 mg/kg, i.p.) 1 hour before (“prophylactic”) or 1 hour after (“therapeutic”) CLP; and capsazepine (Capz) (15 mg/kg, s.c.) or vehicle (DMSO) 30 minutes before CLP. Sham mice were used as controls. Eight hours after CLP or sham operation, (A) lung and (B) plasma SP levels, and (C) lung PPT-A mRNA levels were measured. Results shown are the mean values ± SEM (n = 8–12 mice per group). *P < 0.01 versus sham; **P < 0.01 versus CLP + vehicle.

doi:10.1371/journal.pone.0024535.g005

Figure 6. Effect of PAG and capsazepine on protein levels of cytokines and chemokines in the lungs and liver of septic mice. Mice were randomly given PAG (50 mg/kg, i.p.) 1 hour before (“prophylactic”; PAG+CLP+Vehicle or PAG+CLP+Capz) or 1 hour after (“therapeutic”; CLP+PAG+Vehicle or CLP+PAG+Capz) CLP; and capsazepine (Capz) (15 mg/kg, s.c.) or vehicle (DMSO) 30 minutes before CLP. Sham mice were used as controls. Eight hours after CLP or sham operation, protein levels of (A) TNF-α, (B) IL-1β, (C) IL-6, (D) MIP-1α, and (E) MIP-2 were measured. Results shown are the mean values ± SEM (n = 8–12 mice per group). *P < 0.01 versus sham; **P < 0.01 versus CLP + vehicle; †P < 0.05 versus CLP + vehicle.

doi:10.1371/journal.pone.0024535.g006
NF-κB activation was determined using TransAM NF-κB p65 transcription factor assay kit (Active Motif). The kit consists of a 96-well plate, into which oligonucleotide containing the NF-κB consensus site (5’-GGGACTTTCC-3’) is bound. The active form of NF-κB in the nuclear extract specifically binds to this consensus site and is recognized by a primary antibody specific for the activated form of p65 of NF-κB. An horseradish peroxidase-conjugated secondary antibody provides the basis for the colorimetric quantification. The absorbance of the resulting solution was measured 2 minutes later (450 nm with a reference wavelength of 655 nm), using a 96-well microplate reader (Tecan Systems, Mannedorf, Switzerland). The wild-type consensus oligonucleotide is provided as a competitor for NF-κB binding to monitor the specificity of the assay. Results were expressed as fold increase over the control group.

Western immunoblot

Lung (50 mg) and liver (100 mg) tissues were homogenized at 4°C in 1 ml of radioimmunoprecipitation assay lysis buffer supplemented with 1% protease and 1% phosphatase inhibitor cocktail (Sigma-Aldrich), followed by centrifugation at 14,000 g for 10 minutes at 4°C. Protein concentration in the soluble fraction was determined by the Bradford method. Protein samples (50–100 μg) were separated by SDS-PAGE on Novex Bis-Tris polyacrylamide gels and transferred onto polyvinylidene difluoride membranes by electroblotting in Novex transfer buffer (Invitrogen Life Technologies) containing 20% (v/v) methanol. Membranes were then washed, blocked, and probed overnight at 4°C with rabbit anti-IκBα, phospho-IκBα, ERK1/2, and phospho-ERK1/2 (Cell Signaling Technology, Beverly, MA, USA; 1:1000 dilution for all), followed by secondary detection for 2 hours with an horseradish peroxidase-conjugated, goat anti-rabbit IgG (Santa Cruz Biotechnology, CA, USA; 1:1000 dilution). Membranes were washed and then incubated in SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA) before exposure to X-ray films (LC-Xpose, Pierce Biotechnology). Gels were calibrated by protein kaleidoscope standards (Bio-Rad). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Santa Cruz Biotechnology; 1:2000 dilution) was applied as an internal control to normalize protein loading. The intensity of bands was quantified using LabWorks Image Analysis software (Ultra-Violet Products Ltd., Cambridge, UK).

Figure 7. Effect of PAG and capsazepine on protein levels of adhesion molecules in the lungs and liver of septic mice. Mice were randomly given PAG (50 mg/kg, i.p.) 1 hour before (“prophylactic”; PAG+CLP+Vehicle or PAG+CLP+Capz) or 1 hour after (“therapeutic”; CLP+PAG+Vehicle or CLP+PAG+Capz) CLP; and capsazepine (Capz) (15 mg/kg, s.c.) or vehicle (DMSO) 30 minutes before CLP. Sham mice were used as controls. Eight hours after CLP or sham operation, protein levels of (A) P-selectin, (B) E-selectin, (C) ICAM-1, and (D) VCAM-1 were measured. Results shown are the mean values ± SEM (n = 8–12 mice per group). *P<0.01 versus sham; **P<0.01 versus CLP+vehicle.

doi:10.1371/journal.pone.0024535.g007
A

Plasma ALT activity (IU/L)

Sham  CLP  CLP+ Vehicle  CLP+ Vehicle  CLP+ Capz  CLP+ Vehicle  CLP+ Capz

B

Plasma AST activity (IU/L)

Sham  CLP  CLP+ Vehicle  CLP+ Vehicle  CLP+ Capz  CLP+ Vehicle  CLP+ Capz

C

Pulmonary edema (Wet/dry weight ratio)

Sham  CLP  CLP+ Vehicle  CLP+ Vehicle  CLP+ Capz  CLP+ Vehicle  CLP+ Capz
ratios were measured. Results shown are the mean values ± SEM (n = 10–15 mice per group). *P<0.01 versus sham; **P<0.01 versus CLP vehicle.

doi:10.1371/journal.pone.0024535.g008

Results

Capsazepine attenuates endogenous SP concentrations in both septic and septic mice administered with NaHS

The concentration of SP in both lung (Fig. 1A) and plasma (Fig. 1B) was significantly increased after induction of sepsis. Densitometric analysis of PCR products on agarose gel showed that pulmonary PPT-A mRNA expression correlated well with protein levels (Fig. 1C). Administration of capsazepine significantly suppressed lung (Fig. 1A) and plasma (Fig. 1B) SP levels. Consistently, transcriptional level of PPT-A gene expression was markedly reduced upon treatment with capsazepine (Fig. 1C). Administration of NaHS resulted in a further rise in the pulmonary (Fig. 1A) and plasma (Fig. 1B) SP levels in sepsis. Likewise, pulmonary mRNA level of PPT-A was significantly elevated in septic mice administered with NaHS. Importantly, in the presence of NaHS, a significant attenuation of endogenous SP concentrations occurred in both lung (Fig. 1A) and plasma (Fig. 1B), consistent with a parallel decrease in pulmonary gene expression for SP (Fig. 1C) in septic mice treated with capsazepine.

The attenuated SP levels correlates with reduced production of proinflammatory molecules in both septic and septic mice administered with NaHS

The protein levels of proinflammatory cytokines, TNF-α, IL-1β, IL-6; chemokines, MIP-1α, MIP-2 (Fig. 2A–E); and adhesion molecules, P-selectin, E-selectin, ICAM-1, and VCAM-1 (Fig. 3A–D) in both lung and liver homogenates showed a marked rise in septic mice as compared with sham mice. All of these were significantly lowered by capsazepine (Fig. 2 and 3). Administration of NaHS in septic mice further enhanced the production of these mediators (Fig. 2 and 3) but capsazepine alleviated them (Fig. 2 and 3). At the transcriptional level, similar trends were observed that correlated well with the protein levels in both septic and septic mice administered with NaHS (data not shown).

Capsazepine protects against MODS in both septic and septic mice administered with NaHS

MODS is recognized to be the ultimate cause of death in patients with sepsis [11]. In an attempt to determine if TRPV1 antagonism by capsazepine is beneficial in preventing MODS, we determined pulmonary edema levels to assess the severity of lung injury [35], and measured plasma ALT and AST activities as indices of hepatic dysfunction [40]. Evidence of lung and liver damage after septic injury was confirmed by the heightened levels of pulmonary edema as indicated by wet-to-dry weight ratio (Fig. 4C), and increased activities of plasma ALT (Fig. 4A) and AST (Fig. 4B), respectively. However, septic mice treated with capsazepine showed significantly lower lung wet-to-dry weight ratio (Fig. 4C), and plasma ALT (Fig. 4A) and AST (Fig. 4B) activities. Administration of NaHS in septic mice further exacerbated these organ injury parameters (Fig. 4A–C) but capsazepine alleviated these deleterious effects (Fig. 4A–C).

Capsazepine has no effect on PAG-mediated attenuation of SP levels in sepsis

Our data showed that prophylactic or therapeutic administration of PAG mitigated both pulmonary (Fig. 5A) and plasma (Fig. 5B) SP levels in sepsis. This is consistent with a parallel decrease in pulmonary gene expression for SP (Fig. 5C) in septic mice received PAG intervention. However, there were no significant differences in both the protein and transcriptional levels of SP in septic mice treated with both PAG and capsazepine when compared to their vehicle control counterparts (Fig. 5A–C).

Inhibition of H2S formation impaired proinflammatory molecules production after septic injury, but capsazepine has no effect on them

We found that both pre- and post-treatment of PAG decreased protein levels of proinflammatory cytokines, TNF-α, IL-1β, IL-6; chemokines, MIP-1α, MIP-2 (Fig. 6A–E); and adhesion molecules, P-selectin, E-selectin, ICAM-1, and VCAM-1 (Fig. 7A–D) in both lung and liver homogenates, as compared with vehicle-injected septic mice that exhibited significant increments in the protein levels of these inflammatory mediators. A similar profile of the mRNA expression for these molecules was also observed (data not shown). However, there were no significant differences in the levels of these inflammatory mediators, at both the protein (Fig. 6 and 7) and transcriptional (data not shown) levels, in septic mice treated with both PAG and capsazepine when compared to those that received PAG only.

Beneficial effects of capsazepine and PAG are not additive in protection against MODS in sepsis

Further evidence of neurogenic contribution of H2S in mediating sepsis-associated MODS was demonstrated by non-significant changes of pulmonary edema levels, plasma ALT and AST activities in septic mice treated with both PAG and capsazepine, as compared with their vehicle control counterparts (Fig. 8A–C). These data demonstrated that absence of H2S alone is sufficient to ameliorate lung and liver dysfunction as indicated by decreased levels of lung wet-to-dry weight ratio (Fig. 8C), and reduced activities of plasma ALT (Fig. 8A) and AST (Fig. 8B), respectively.

Effect of capsazepine on ERK1/2 activation in H2S-induced neurogenic inflammation in sepsis

To investigate the signaling mechanisms by which H2S regulates TRPV1-mediated neurogenic inflammation in sepsis, we evaluated the ERK1/2 and NF-κB signaling pathways, with the usage of capsazepine, using two different and complementary approaches: exogenous administration of NaHS as an H2S donor and inhibition of endogenous H2S formation by PAG. We observed activation of pulmonary and hepatic ERK1/2 in septic mice as compared with sham mice (Fig. 9A). Upon treatment of
H2S, SP and ERK-NF-κB in Polymicrobial Sepsis

A

Lung phospho-ERK1/2

Lung ERK1/2

Liver phospho-ERK1/2

Liver ERK1/2

B

Lung phospho-ERK1/2

Lung ERK1/2

Liver phospho-ERK1/2

Liver ERK1/2

Activation of ERK1/2
(Fold increase over sham)

Sham

CLP

CLP + Vehicle

CLP + Capz

CLP + NaHS

CLP + NaHS + Capz

Sham

CLP

CLP + Vehicle

CLP + Capz

CLP + Prophylactic PAG

CLP + Therapeutic PAG
capsazepine, phosphorylation of ERK$_{1/2}$ in lung and liver were abolished (Fig. 9A). Administration of exogenous NaHS further enhanced tissue ERK$_{1/2}$ activation in sepsis, whereby capsazepine treated, NaHS-injected septic mice revealed significantly reduced lung and liver phospho-ERK$_{1/2}$ expressions (Fig. 9A). Additionally, prophylactic or therapeutic administration of PAG markedly suppressed tissue phosphorylation of ERK$_{1/2}$ in sepsis (Fig. 9B). Interestingly, the status of ERK$_{1/2}$ activation in septic mice administrated with both PAG and capsazepine remained unchanged when compared to those that received PAG only (Fig. 9B).

Effect of capsazepine on IkBz phosphorylation and degradation levels and NF-kB activity in H$_2$S-induced neurogenic inflammation in sepsis

Subsequently, we analyzed whether ERK$_{1/2}$ signaling leads to activation of inhibitory IkBz (IkBz) and NF-kB in H$_2$S-induced neurogenic inflammation in sepsis. Capsazepine greatly reduced pulmonary and hepatic levels of phospho-IkBz in septic mice as compared to the elevated levels detected in untreated septic mice (Fig. 10A). Consistently, phospho-IkBz levels in the lungs and liver of septic mice injected with NaHS were greatly enhanced; while these levels were significantly suppressed by capsazepine (Fig. 10A). In contrast, PAG intervention drastically reduced lung and liver phospho-IkBz levels in sepsis and capsazepine has no effect on them (Fig. 10B).

More importantly, assessment of IkBz degradation displayed a similar profile that correlated well with the levels of phospho-IkBz, with untreated septic mice showing a significant reduction in lung and liver IkBz levels in comparison with the heightened levels exhibited in capsazepine treated septic mice (Fig. 10C). Likewise, the expression levels of IkBz in lung and liver tissues from septic mice injected with NaHS were again lowered; while these levels were elevated with capsazepine (Fig. 10C). Additionally, treatment with PAG markedly enhanced tissue IkBz expression levels in comparison to their vehicle control counterparts, suggesting an obvious inhibition of IkBz degradation (Fig. 10D). However, treatment of capsazepine failed to further modulate these levels (Fig. 10D).

Next, we examined whether phosphorylation and degradation of IkBz result in NF-kB nuclear translocation. Our results showed that capsazepine significantly decreased the DNA-binding activity of nuclear NF-kB in the lungs and liver of septic mice (Fig. 11A). Exogenous administration of NaHS further amplified the activation of NF-kB while treatment with capsazepine significantly disrupted the activity of NF-kB (Fig. 11A). In the absence of endogenous H$_2$S, the DNA-binding activity of nuclear NF-kB was greatly reduced (Fig. 11B). As anticipated, we noticed no difference in lung and liver NF-kB activation from septic mice treated with both PAG and capsazepine as compared with their vehicle control counterparts (Fig. 11B).

Finally, to examine if ERK$_{1/2}$ activation occurs upstream of NF-kB, we measured tissue NF-kB activity in the presence of PD98059, a potent and selective antagonist of MEK-1 that is the upstream kinase of ERK$_{1/2}$ [28,29]. Our results showed that PD98059 significantly decreased the DNA-binding activity of nuclear NF-kB in the lungs and liver of septic mice (Fig. 11C). In the presence of NaHS, a marked attenuation of tissue NF-kB activation by PD98059 was observed in sepsis, thus providing convincing evidence that ERK$_{1/2}$ activation occurs upstream of NF-kB (Fig. 11C).

Discussion

Sepsis remains an important global healthcare problem even in the modern era of critical care management. Thus, identifying endogenous neural elements that modulate H$_2$S-induced neurogenic inflammation in sepsis and elucidating the underlying molecular mechanisms by which this occurs are of paramount importance. Our previous study demonstrated that H$_2$S promotes TRPV1-mediated neurogenic inflammation in polymicrobial sepsis [10]. In the present study, we showed that overproduction of SP significantly increases the severity of sepsis in a TRPV1-dependent manner; and that this phenomenon occurred under the influences of the proinflammatory effects of H$_2$S. Additionally, we found that the underlying signal transduction pathway by which H$_2$S, SP and TRPV1 interact to instigate neural inflammatory processes involve activation of the ERK-NF-kB pathway.

In recent years, the role of SP in the regulation of inflammatory conditions in lipopolysaccharide-evoked endotoxemia, CLP-administered sepsis, as well as human sepsis and septic shock has been suggested [41,42,43]. Of even greater significance, H$_2$S has been shown to implicate in neurogenic inflammation mediated by SP and TRPV1. By stimulating TRPV1-expressing sensory neurons, H$_2$S provoked the release of SP from isolated guinea pig airways [5]. Likewise, heightened circulatory level of SP was observed in normal mice administrated with NaHS [19]. Notably, in both studies, SP levels were greatly attenuated by capsaicin desensitization or by capsazepine administration. Besides, H$_2$S induced contraction in rat urinary bladder via a neurogenic mechanism that involved TRPV1 stimulation with consequent release of tachykinin [44,45]. Furthermore, endogenous H$_2$S was found to modulate sepsis-associated lung injury and caraculine-induced acute pancreatitis through upregulation of SP in the lungs and pancreas, respectively [43,46]. Here, we show that H$_2$S regulates TRPV1-mediated neurogenic inflammation in sepsis through upregulation of pulmonary and plasma SP. Our findings are consistent with earlier observations and reinforce the essential role of SP, and its interaction with H$_2$S. It must be noted, however, that with the usage of capsazepine, a synthetic competitive antagonist of TRPV1; our results provide the first pharmacological evidence that H$_2$S provokes tachykinin-mediated neurogenic inflammatory responses involving SP in sepsis in a TRPV1-dependent manner.

In the first report showing the relations between H$_2$S and infection, endogenous vascular H$_2$S content was increased in rats with septic and endotoxic shock. It was suggested that excess H$_2$S production was involved in pathophysiological processes during shock [9]. Consistently, our previous study has shown that CLP-induced sepsis significantly increased the plasma H$_2$S level and the liver CSE activity in septic mice. Prophylactic and therapeutic administration of PAG in septic mice completely abolished the
Figure 10. Effect of NaHS or PAG and capsazepine on IκBα phosphorylation and degradation levels in the lungs and liver of septic mice. Mice were randomly given NaHS (10 mg/kg, i.p.) at the same time of CLP or PAG (50 mg/kg, i.p.) 1 hour before (“prophylactic”) or 1 hour after (“therapeutic”) CLP; and capsazepine (Capz) (15 mg/kg, s.c.) or vehicle (DMSO) 30 minutes before CLP. Sham mice were used as controls. Eight hours after CLP or sham operation, effect of NaHS or PAG and capsazepine on (A and B) phospho-IκBα and (C and D) IκBα expression levels were measured.
CSE synthesizing activity in the liver, thereby leading to a significant reduction in plasma H$_2$S level [10]. Furthermore, capsazepine has no effect on endogenous generation of H$_2$S in sepsis. Employing the same experimental approach, it was demonstrated that hepatic CSE activity and plasma H$_2$S level in septic mice treated with capsazepine and those treated with both PAG and capsazepine were comparable to their vehicle control counterparts [10]. Here, we report that capsazepine significantly attenuated endogenous SP levels in both septic and septic mice administrated with NaHS. Moreover, capsazepine has no effect on PAG-mediated attenuation of SP level in sepsis. Taken together, these findings suggest that endogenous H$_2$S signaled via TRPV1, which resulted in the subsequent release of SP, thereby allowing SP to elicit its potent proinflammatory effects in sepsis. It is worth mentioning that TRPV1 is broadly expressed in all “port of entry” tissues such as the skin, gut, airway and conjunctiva [2,47], hence we restricted measurement of SP to the lungs. Nevertheless, a systemic role of SP via the circulatory system was investigated in this study to substantiate its generalized role in mediating neurogenic inflammation in sepsis.

SIRS and MODS are major hallmarks of sepsis. Excessive synthesis of inflammatory molecules in organs remote from the initial insult synergistically interact to mediate tissue damage, followed by cardiovascular collapse, MODS, and death of the host [11]. The current study revealed that pharmacological blockade of TRPV1 markedly reduced pulmonary and hepatic levels of IL-1β, IL-6, TNF-α, MIP-1α, MIP-2, P-selectin, E-selectin, VCAM-1 and ICAM-1 in both septic and septic mice administered with NaHS. Likewise, a significant protection against lung injury and liver dysfunction, as indicated by pulmonary edema and serum ALT and AST activities respectively, was conferred by capsazepine. Importantly, both observations corresponded with concurrent attenuation of SP level, suggesting that H$_2$S stimulation of TRPV1 and the subsequent release of SP can induce upregulation of proinflammatory mediators that implicated in SIRS and work in conjunction with them to initiate MODS. The observed protection against MODS was further associated with improved survival benefits, as capsazepine has been previously reported to reduce mortality in sepsis even in the presence of NaHS [10]. To this regard, it should be noted that lung and liver were chosen for investigation primarily because lungs are often the first organ to succumb post systemic insults, and liver represents an important organ not only in host defense [48], but in addition that CSE is highly expressed in hepatocytes [49]. Collectively, our results established H$_2$S stimulation of TRPV1 and the downstream release of SP as a key element in the transition of infection and SIRS to MODS in sepsis, and identified TRPV1 antagonist as a possible therapeutic target for the treatment of polymicrobial sepsis.

In contrast, beneficial effects of TRPV1 antagonism in ameliorating SIRS and MODS in sepsis were diminished when endogenous synthesis of H$_2$S was blocked as seen in septic mice received PAG intervention as compared to the same mice treated with capsazepine. It appears that inhibition of H$_2$S generation alone is sufficient to account for these observations, and that the presence or absence of TRPV1 antagonism makes no difference. Nonetheless, with regard to the inhibitory effects of CSE synthesizing activity, it should be underlined that the effects of PAG alone seem not to be a consequence of blood pressure changes, given that at the doses used PAG did not significantly change blood pressure [50]. Besides, the effect of capsazepine is not entirely secondary to an enhanced formation of H$_2$S. Since sepsis is a multifactorial disease, other inflammatory mediators may stimulate TRPV1 and trigger the release of SP. Nevertheless, we ascertained from our data that sepsis has a significant sensory neurogenic component that involved SP and mediated by H$_2$S in a TRPV1-dependent manner.

Although convincing data have suggested the relevance of ERK$_{1/2}$ signaling to H$_2$S, their association with TRPV1 remains unknown [18,51,52,53]. In the current study, we showed that pharmacological antagonism of TRPV1 inhibits phosphorylation of ERK$_{1/2}$ and IκBα, concurrent with inhibition of IκBα degradation and DNA binding activity of nuclear NF-κB, in both septic and septic mice administrated with NaHS. Conversely, in the absence of H$_2$S, capsazepine has no effect on PAG-mediated suppression of these parameters in sepsis. These findings not only indicate that H$_2$S, SP and TRPV1 interact to instigate neurogenic inflammation in sepsis through activation of the ERK-NF-κB pathway, but also added to the growing evidence about the involvement of ERK$_{1/2}$ in regulating the activity of NF-κB [14,15], and the potential association between H$_2$S and ERK-NF-κB [18,51,52,53]. Furthermore, the observed suppression of tissue NF-κB activation in the presence of PD98059, a selective inhibitor of MEK-1 that is the upstream kinase of ERK$_{1/2}$, directly ascertained that ERK$_{1/2}$ signaling occurs upstream of NF-κB activation. Reinforcing our findings that H$_2$S induces TRPV1-mediated neurogenic inflammation through activation of the ERK-NF-κB pathway, H$_2$S has been reported previously, using specific inhibitors for ERK and NF-κB, to alleviate tissue inflammatory cytokines and chemokines production in septic mice and normal mice injected with NaHS, respectively [18,54]. Nevertheless, H$_2$S has been linked to a number of other pathways [55,56,57,58], hence, alternative mechanisms may exist which act in synergism with ERK signaling cascade to modulate the activity of NF-κB in sepsis. Additionally, since polyclonal antibody against p65 subunit was employed to investigate the activity of NF-κB, our results demonstrate that NF-κB dimers containing p65 may play a crucial role in H$_2$S-provoked inflammation and injury response seen in this study.

Our findings suggest a proinflammatory role of H$_2$S in sepsis, however, a recent paper by Spiller et al. [59] reports the anti-inflammatory properties of H$_2$S. The inconsistency may be a result of the dose of NaHS used. We used NaHS at a dose of 10 mg/kg, which increased plasma concentration of H$_2$S significantly and caused obvious lung and liver inflammation [10,19,26] whereas study by Spiller et al. used three doses of NaHS (10, 30, or 100 μmol/kg) that approximated the physiological concentrations of H$_2$S. Importantly, it has been suggested that low (physiological) concentrations of H$_2$S tend to be cytoprotective and anti-inflammatory while higher concentrations are likely to be cytotoxic and proinflammatory [6]. In addition, experimental differences that include health status, age, and gender of the animals; commercial sources of NaHS and PAG; and level of severity of sepsis may explain the opposing findings between us and them.

Although the present study offers the possibility that H$_2$S may activate TRPV1, the precise molecular site of action and the molecular mechanisms underlying it remain unknown. It could be possible that the neuropeptide release observed with H$_2$S is via an
H2S, SP and ERK-NF-κB in Polymicrobial Sepsis

A

B

C

7

6

5

4

3

2

1

0

Sham

CLP

CLP + Vehicle

CLP + Capz

CLP + NaHS + Vehicle

CLP + NaHS + Capz

0

1

2

3

4

5

6

7

Sham

CLP

CLP + Vehicle

CLP + Capz

CLP + NaHS + Vehicle

CLP + NaHS + Capz

Sham

CLP

CLP + Vehicle

CLP + Capz

CLP + NaHS + Vehicle

CLP + NaHS + Capz

Prophylactic PAG

Therapeutic PAG

Lung • Liver

Lung • Liver

Lung • Liver

Tissue NF-κB activity
(Fold increase over sham)

Tissue NF-κB activity
(Fold increase over sham)

Tissue NF-κB activity
(Fold increase over sham)
indirect activation of TRPV1 through other endogenous mediators evoked by H$_2$S. Furthermore, the specific cell types that respond to H$_2$S stimulation of TRPV1 activation, as well as the types of cells that bind to SP and mediate ERK-NF-κB signaling, remain to be identified. Molecular mechanisms that lead to reduced ERK$_{1/2}$ phosphorylation and NF-κB activation with PAG are issues deserving further studies. Finally, investigation on the role of H$_2$S in pathological conditions has been hampered by the paucity of specific pharmacological tools. Most irreversible inhibitors of CSE, including PAG, are of low potency, of low selectivity and of limited cell-membrane permeability that also non-specifically interfere with other pyridoxal-5'-phosphate-dependent enzymes [6,8]. It is clear that newer and more promising chemical tools are required to probe deeper the complex biological roles of H$_2$S.

In conclusion, we propose that H$_2$S regulates TRPV1-mediated neurogenic inflammation in polymicrobial sepsis through enhancement of SP production and activation of the ERK-NF-κB pathway. Importantly, the H$_2$S-TRPV1-SP-ERK$_{1/2}$-IκBα-NF-κB signal transduction pathway contributes to SIRS and MODS in sepsis (Fig. 12). Collectively, our results contribute to a better understanding of the precise mechanism underlying the proinflammatory effects of H$_2$S in the pathophysiology of sepsis in a TRPV1 relevance context and provide further insight into the development of new therapeutic intervention for sepsis and other inflammatory pathologies.

Figure 11. Effect of NaHS or PAG and capsazepine and effect of NaHS and PD98059 on NF-κB activation in nuclear extracts of lung and liver tissues in septic mice. Mice were randomly given NaHS (10 mg/kg, i.p.) at the same time of CLP or PAG (50 mg/kg, i.p.) 1 hour before (“prophylactic”) or 1 hour after (“therapeutic”) CLP; and capsazepine (Capz) (15 mg/kg, s.c.) or vehicle (DMSO) 30 minutes before CLP. Some mice were injected PD98059 (10 mg/kg, i.p.) or vehicle (DMSO) 1 hour before CLP. Sham mice were used as controls. Eight hours after CLP or sham operation, effect of (A) NaHS or (B) PAG and capsazepine and (C) effect of NaHS and PAG on the DNA-binding activity of NF-κB were measured. Results shown are the mean values ± SEM (n = 8–12 mice per group). *P < 0.01 versus sham; **P < 0.01 versus CLP + vehicle; †P < 0.01 versus CLP + NaHS + vehicle. ‡P < 0.01 versus CLP + NaHS + vehicle.

doi:10.1371/journal.pone.0024535.g011

Figure 12. Schematic summary of signaling events in H$_2$S-induced neurogenic inflammation in a murine model of polymicrobial sepsis. H$_2$S has been demonstrated to be overproduced in sepsis. H$_2$S stimulation of TRPV1 and the downstream release of SP lead to the activation of ERK$_{1/2}$, which subsequently induces the phosphorylation and degradation of IκBα, as well as the translocation and activation of NF-κB, thereby leading to SIRS and MODS characteristic of severe sepsis. ← indicates exogenous administration; † indicates inhibition.

doi:10.1371/journal.pone.0024535.g012
Acknowledgments
The authors are grateful to NUS Graduate School for Integrative Sciences and Engineering for providing Seah-Fang Ang scholarship for graduate studies.

References
1. Sternberg EM (2006) Neural regulation of innate immunity: a coordinated nonspastic host response to pathogens. Nat Rev Immunol 6: 318–329.
2. O’Connor TM, O’Connel J, O’Brien DI, Goode T, Bredin CP, et al. (2004) The role of substance P in inflammatory disease. J Cell Physiol 201: 167–180.
3. Richardson JD, Vasko MR (2002) Cellular mechanisms of neurogenic inflammation. J Pharmacol Exp Ther 302: 839–845.
4. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, et al. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389: 816–824.
5. Trevisani M, Patacchini R, Nicoletti P, Gatti R, Gazzieri D, et al. (2005) Hydrogen sulfide causes vanilloid receptor-1-mediated neurogenic inflammation in the airways. Br J Pharmacol 145: 1123–1131.
6. Szabo C (2007) Hydrogen sulphide and its therapeutic potential. Nat Rev Drug Discov 6: 917–936.
7. Bhatia M (2005) Hydrogen sulfide as a vasodilator. IUBMB Life 57: 603–606.
8. Li L, Moore PK (2008) Putative biological roles of hydrogen sulfide in health and disease: Not so foreign a friend? Trends Pharmacol Sci 29: 84–90.
9. Hui Y, Du J, Tang C, Bin G, Jiang H (2003) Changes in arterial hydrogen sulfide (H2S) content during septic shock and endotoxin shock in rats. J Infect 43: 155–160.
10. Ang SF, Moochhala SM, Bhatia M (2010) Hydrogen sulfide promotes transient receptor potential vanilloid 1-mediated neurogenic inflammation in polymicrobial sepsis. Crit Care Med 38: 619–628.
11. Cohen J (2002) The immunopathogenesis of sepsis. Nature 420: 885–891.
12. Bredin CP, O’Connor TM, Goode T, O’Brien DI, Bredin SP, et al. (2004) Temporal control of NF-kappaB activation by ERK differentially regulates interleukin-beta-induced gene expression. J Biol Chem 279: 1322–1329.
13. Roux PP, Bienen J (2004) ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev 68: 320–344.
14. Pham H, Lee C, Cho HS, Kim Y, Yan Y, et al. (2007) Inhibitory effect of olopatadine on nitric oxide production and activation of NF-kappaB/NFAT MAP kinases in lipopolysaccharide-treated RAW 264.7 cells. Eur J Pharmacol 556: 181–189.
15. Dumitru CD, Cee JD, Taasian S, Kontoyiannis D, Stamatakis K, et al. (2000) TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell 105: 1071–1083.
16. Zhang H, Moochhala SM, Bhatia M (2008) Endogenous hydrogen sulfide regulates inflammatory response by activating the ERK pathway in polymicrobial sepsis. J Immunol 181: 4320–4331.
17. Di Paola R, Galuppo M, Mazzone E, Paterniti I, Bramanti P, et al. (2010) PD08059, a specific MAP kinase inhibitor, attenuates multiple organ dysfunction syndrome/failure (MODS) induced by zymosan in mice. Pharmacol Res 61: 173–187.
18. Zuege D, Suzuki S, Berthiaume Y (1996) Increase of lung sodium-potassium- ATPase activity during recovery from high-permeability pulmonary edema. Am J Physiol 271: L197–209.
19. Pitter JF, Wierer-Kronlich JP, McElroy MC, Folkeson GH, Matthay MA (1994) Stimulation of lung epithelial liquid clearance by endogenous release of calcitonin gene-related peptide in septic shock in anesthetized rats. J Clin Invest 94: 663–671.
20. Pitter JF, Griffiths MJ, Greuter T, Kaminski N, Dalon SL, et al. (2001) TGF-beta is a critical mediator of acute lung injury. J Clin Invest 107: 1537–1544.
21. Kew MC (2000) Serum aluminosterase concentration as evidence of hepato-cellular damage. Lancet 355: 591–592.
22. Ng SW, Zhang H, Hegde A, Bhatia M (2008) Role of preprotachykinin-A gene products on multiple organ injury in LPS-induced endotoxemia. J Leukoc Biol 83: 281–295.
23. Beer S, Weighardt H, Emmanouilidi H, Harzerdtert MM, Matevosian E, et al. (2002) Systemic neuropeptide levels as predictive indicators for lethal outcome in patients with postoperative sepsis. Crit Care Med 30: 1794–1798.
24. Zhang H, Hegde A, Ng SW, Adhikari S, Moochhala SM, et al. (2007) Hydrogen sulfide up-regulates substance P in polymicrobial sepsis-associated lung injury. J Immunol 179: 4153–4160.
25. Patacchini R, Santioli P, Giuliani S, Maggi CA (2005) Pharmacological investigation of hydrogen sulfide (H2S) contractile activity in rat detrusor muscle. Eur J Pharmacol 530: 171–177.
26. Patacchini R, Santioli P, Giuliani S, Maggi CA (2004) Hydrogen sulfide (H2S) stimulates capsaicin-sensitive primary afferent neurones in the rat urinary bladder. Br J Pharmacol 142: 31–41.
27. Bhatia M, Sithikaruvila JN, Ng SW, Tamizhevli R, Moochhala SM (2008) Provlimflammatory effects of hydrogen sulfide on substance P in caerulein-induced acute pancreatitis. J Cell Mol Med 12: 580–590.
28. Veronesi B, Oortjes M (2006) The TRPV1 receptor: target of toxicants and therapeutics. Toxicol Sci 89: 1–13.
29. Wang P, Chaudhry IH (1996) Mechanism of hepato-cellular dysfunction during hyperdynamic sepsis. Am J Physiol 270: R927–933.
30. Mudd SH, Finkelstein JD, Irreverre F, Laster I, (1963) Transfusion in man. Microsacs and tissue distributions of three enzymes of the pathway. J Biol Chem 240: 3492–3499.
31. Mok YY, Atan MS, Yoke Ping C, Zhong Jing W, Bhatia M, et al. (2004) Role of histamine in inflammatory response in murine acute pancreatitis in the rat. J Pathol 202: 174–182.
32. Di Paola R, Crisafulli C, Mazzone E, Genovese T, Paterniti R, et al. (2009) Effect of PD98059, a selective MAPK3/MAPK1 inhibitor, on acute lung injury in mice. Int J Immunopharmacol 22: 937–950.
33. Zuege D, Suzuki S, Berthiaume Y (1996) Increase of lung sodium-potassium-ATPase activity during recovery from high-permeability pulmonary edema. Am J Physiol 271: L197–209.

Author Contributions
Conceived and designed the experiments: SMM PAMB. Performed the experiments: S-FA. Analyzed the data: S-FA MBM. Contributed reagents/materials/analysis tools: SMM PAMB. Wrote the paper: S-FA SMM PAMB.
Zhang H, Zhi L, Moochhala S, Moore PK, Bhatia M (2007) Hydrogen sulfide acts as an inflammatory mediator in cecal ligation and puncture-induced sepsis in mice by upregulating the production of cytokines and chemokines via NF-kappaB. Am J Physiol Lung Cell Mol Physiol 292: L960–971.

Yang G, Sun X, Wang R (2004) Hydrogen sulfide-induced apoptosis of human aorta smooth muscle cells via the activation of mitogen-activated protein kinases and caspase-3. FASEB J 18: 1782–1784.

Yong QC, Choo CH, Tan BH, Law CM, Bian JS (2010) Effect of hydrogen sulfide on intracellular calcium homeostasis in neuronal cells. Neurochem Int 56: 508–515.

Lee SW, Hu YS, Hu LF, Lu Q, Dasee GS, et al. (2006) Hydrogen sulphide regulates calcium homeostasis in microglial cells. Glia 54: 116–124.

Tamizh selvi R, Sun J, Koh YH, Bhatia M (2009) Effect of hydrogen sulfide on the phosphatidylinositol 3-kinase-protein kinase B pathway and on caerulein-induced cytokine production in isolated mouse pancreatic acinar cells. J Pharmacol Exp Ther 329: 1166–1177.

Spiller F, Orrico MI, Nascimento DC, Czaikoski PG, Souto FO, et al. (2010) Hydrogen sulfide improves neutrophil migration and survival in sepsis via K+ATP channel activation. Am J Respir Crit Care Med 182: 360–368.