Explorations of the therapeutic potential of influencing metabolism during critical illness

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Hydrogen sulfide donor NaHS reduces organ injury in pneumosepsis, associated with improved bio–energetic status

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Submitted
Abstract

Sepsis is characterized by a generalized inflammatory response and organ failure, associated with mitochondrial dysfunction. Hydrogen sulfide donor NaHS is able to reduce metabolism and can preserve mitochondrial morphology and function. Rats were challenged with live *Streptococcus pneumoniae* or saline and infused with NaHS (36 µmol/kg/h) or vehicle. Lung injury markers were measured as well as mitochondrial respiration, viability and biogenesis. Infusion of NaHS reduced heart rate and body temperature, indicative of a hypo–metabolic state. NaHS infusion reduced sepsis–related organ injury, associated with a reversal of a fall in active oxidative phosphorylation with a concomitant decrease in ATP levels and ATP/ADP ratio. Preservation of mitochondrial respiration was associated with increased mitochondrial expression of α–tubulin and protein kinase C–ε, which acts as regulators of respiration. Mitochondrial damage was decreased, as suggested by a reduction in mitochondrial DNA leakage in the lung during pneumosepsis, associated with a reduction in organ reactive oxygen damage. Also, NaHS initiated mitochondrial biogenesis by upregulating peroxisome proliferator–activated receptor–γ coactivator 1α, with a subsequent increase in transcription of mitochondrial respiratory subunits. These findings may indicate that the ability of NaHS to reduce organ injury in pneumosepsis is due to the preservation of oxidative phosphorylation and thereby ATP synthesis, via a mechanism which improves mitochondrial respiration and promotes mitochondrial biogenesis.
Introduction

*Streptococcus pneumoniae* is the leading cause of community acquired pneumonia in patients that require admission to the intensive care unit and mechanical ventilation (1). Infection with *S. pneumoniae* triggers an intense inflammatory reaction in the lung, which can lead to sepsis and multiple organ failure. The kidney is the first organ to fail, whereas liver failure occurs later during the course of the disease (1). Supportive treatment aims to enhance substrate and oxygen delivery to tissues to produce adenosine triphosphate (ATP), thereby preserving the bio-energetic status of organs (2). However, despite adequate supportive treatment, mortality of sepsis remains high (3).

The mechanisms which underlie sepsis–induced organ injury are complex. In response to intruding pathogens, immune cells produce an enormous amount of pro-inflammatory cyto– and chemokines (2). This inflammatory response can directly damage mitochondrial DNA, lipids and respiratory complexes, thereby inhibiting oxidative phosphorylation (4) and diminishing ATP availability in organs (2). In sepsis, mitochondrial dysfunction is associated with adverse outcome (5). Influencing mitochondrial substrate utilization was shown to improve outcome in sepsis (6–8), which may be due to preserved mitochondrial structure and function (9), thereby preserving local ATP levels.

Regulation of cellular metabolism is complex and involves various nuclear transcription factors (10). Upon stress stimulation, peroxisome proliferator–activated receptor–γ coactivator (PGC)–1α rapidly induces the upregulation of transcription factors resulting in transcription of mitochondrial (mt) DNA, with subsequent formation of several subunits of respiratory complexes inside the mitochondria, including cytochrome c oxidase (COX) 1, NADH1 and NADH6 (10). Expansion of respiratory complexes increases oxidative phosphorylation and thereby ATP production. ATP availability is regulated by VDAC, a voltage–dependent porin, embedded in the outer membrane of the mitochondria (11). ATP is transported from the matrix to the cytosol through VDAC in exchange for ADP and phosphate. The function of VDAC is partly regulated by α–tubulin and protein kinase C (PKC)–ε, which can induce closure of VDAC (12), thereby reducing the exchange of ATP with ADP to the cytosol.

Hydrogen sulfide (H₂S) is an inhibitor of mitochondrial respiration (13) and has been shown to induce a shift in mitochondrial substrate utilization (14), to improve mitochondrial structure and function during ischemic injury (13) and to maintain ATP production under hypoxic conditions (15). Also, H₂S has anti–inflammatory properties (16). In sufficient doses, H₂S can induce a ‘hibernation–like state’, characterized by hypothermia and low CO₂ production and O₂ consumption (17). Infusion of H₂S donor NaHS has been shown to reduce lung inflammation in a rat model of sterile lung injury (18). Furthermore, bolus NaHS improved survival when injected simultaneously at the time of induction of polymicrobial sepsis (19) in mice. However, NaHS has a short half life (16). The effect of extended infusion
of high doses of NaHS during sepsis is unknown. Also, the mechanisms of the effects of NaHS on mitochondrial function are not known. In the present study, we hypothesized that NaHS protects against organ damage in a rat model of pneumococcal pneumosepsis. Furthermore, we studied the pathways by which $H_2S$ regulates mitochondrial function.

**Methods**

The study was approved by the animal care and use committee of the Academic Medical Centre, Amsterdam, the Netherlands. Animal procedures were carried out in compliance with Institutional Standards for Human Care and Use of Animal Laboratory Animals.

*Induction of pneumonia*

Rats (Sprague Dawley ± 350 g, Harlan, The Hague, The Netherlands) were intratracheally inoculated with $\sim 6 \times 10^6$ CFU of aerosolized *S. pneumoniae* serotype 3 (ATCC 6303; Rockville, MD, USA) using a trans–oral miniature nebulizer under light anesthesia (97% oxygen with 3% isoflurane). Controls received saline. Supplemental fluids were given after inoculation and every 24 hours by intraperitoneal injection of 10 ml/kg of lactated Ringer’s solution.

*$H_2S$ donor*

Preparations of a $H_2S$ donor were made fresh on the day of the experiments as described before (18). NaHS was infused at 36 µmol/kg/h intravenously.

*Experimental protocol and groups*

Two days after inoculation with *S. pneumoniae* rats were anesthetized with intraperitoneal injection of a mix (0.15 ml/100g body weight) containing 90 mg/kg ketamine, 0.5 mg/kg medetomidine and 0.05 mg/kg atropine. Anesthesia was maintained by infusion of 50 mg/kg ketamine at 0.5 ml/100g/hr. Bicarbonate (8.4%) was administered at 1 ml/100g/hr to maintain normal acid–base balance (20). A tracheotomy was performed, after which a metal canule was connected to a ventilator (Servo 900C, Siemens, Sweden). Ventilator settings were determined in pilot experiments aiming at normo–pH (7.35–7.45) and tidal volumes of $\sim 7.5$ ml/kg (18). Detailed ventilatory settings are described in supplementary data. Hemodynamic monitoring was done by a carotid artery catheter connected to a monitor. Urine was collected. After baseline measurements, the animals were randomized to infusion with saline or NaHS, with equal volume load. In the saline control groups, body temperature was maintained at 37°C.
Exsanguination and assays
The rats were sacrificed after 4 hours of mechanical ventilation. Lungs were removed en block. BALF was obtained by flushing the left lung (3 x 2.5 ml saline) after ligation of the right lung. Cell counts were determined using a hemocytometer (Z2 Coulter Particle Counter, Beckman Coulter; Florida, USA) and cell differentiation were done on Giemsa–stained cytospins in bronchoalveolar lavage fluid (BALF). Interleukin (IL)–1β, IL–6, TNF–α and CINC3 (R&D Systems; Abingdon, United Kingdom) were measured in BALF and plasma. Alkaline phosphatase (AF) levels were measured by a commercially available kit (Sigma Aldrich, St. Louis, MO) in BALF supernatant using a Hitachi analyzer (Roche BV, Mannheim, Germany). Plasma levels of aspartate amino–transaminase and alanine–transaminase were measured using standard enzymatic methods at 37°C and creatinine by enzymatic PAP (Roche Diagnostics, the Netherlands). To measure protein carbonyls, lung and liver sample (±200 mg) were prepared according to the manufacturer’s instruction (Cayman chemical company, Ann Arbor, Michigan, USA). Glomerular filtration rate was calculated by ([creatinine] urine x urine volume in 24 hours) / [creatinine] plasma.

Bacterial outgrowth, wet weight and histopathology
Approximately 0.5 g of lung, liver and spleen tissues were removed, diluted 1:4 in sterile saline and homogenized. The number of colonizing forming units (CFU) was determined by performing 10–fold dilutions in blood, BALF and organ homogenates on blood agar plates, after 24 hours of incubation at 37°C with 5% CO₂. Right lung top, liver and kidney were hematoxylin–eosin stained and analyzed by a pathologist who was blinded for group identity. Interstitial inflammation, endothelialitis, bronchitis, edema and pleuritis were scored on a scale of 0 – 4: 0 for normal lungs, 1 for <25% lung involvement, 2 for 25 – 50% involvement, 3 for 50–75% involvement and 4 for >75% lung involvement. Total histology score is the sum score of all parameters. The remaining right lobes were weighted to determine wet weight, as was the right kidney.

Mitochondrial viability measurements
Part of the liver was removed by modified freeze clamping and placed in liquid nitrogen for ATP and adenosine diphosphate (ADP) measurements and part was placed in isolation buffer. This was done before exsanguination to ensure mitochondrial viability and minimize ATP degradation. Mitochondria were isolated by differential centrifugation steps and respiration was measured polarographically at 37°C using a respiratory system (System S 200A, Strathkelvin Instruments, Glasgow, Scotland). ATP and ADP levels were analysed by HPLC method. The expression of VDAC and its regulators, including α–tubulin and phosphor PKC–ε were measured at protein level in the mitochondrial fraction by western blot. Expression of VDAC and α–tubulin was normalized to expression of prohibitin. Expression of
phosphorylated PKCε is shown as a ratio to total PKCε. Expression of PGC1–α, COX1, NADH1 and NADH6 were measured at mRNA level in liver and lung tissue as well as in plasma and BALF. See supplemental data for more detailed description.

**Statistical analysis**

Data are expressed as mean with SD in the tables and in the figures as mean with SEM (to enhance visual clarity). Bacterial outgrowth data was analyzed by Mann–Whitney U test or unpaired t–test according to data distribution. Intergroup differences were analyzed by analysis of variance (ANOVA) and Bonferroni’s post–hoc test, or by a Kruskal Wallis test with Mann–Whitney U test dependent on data distribution. A p value of < 0.05 was considered significant. Statistical analyses were done using Prism (Graphpad Prism 5, CA, USA).

**Results**

*S. pneumoniae resulted in severe pneumosepsis, characterized by lung injury, systemic inflammation, organ injury and low bio–energetic status*

After inoculation with *S. pneumoniae* but before NaHS infusion, the mortality rate was 11%. These animals were not included. The remaining animals were randomized to NaHS or vehicle infusion. Mean arterial pressure remained above 70 mmHg (table 1). Inoculation resulted in severe macroscopic pneumonia. Hematogenic spread of bacteria was demonstrated by positive cultures of liver or spleen in all animals (figure S1).

Pneumonia increased lung wet weight and bronchoalveolar lavage fluid (BALF) protein content compared to non–infected controls (p<0.05, figure 1), accompanied by an enhanced pulmonary cell– and neutrophil influx (p<0.05, figure 1), increased BALF levels of IL–1β, IL–6, TNF–α and CINC3 (p<0.05 for all, figure 1) in addition to lung histopathological abnormalities (p<0.05, figure 2). Pneumonia resulted in increased levels of AF (48.5±10.4 vs. 29.1±3.7 U/L, p<0.05) in the BALF compared to non–infected controls, indicating cell damage. Pulmonary injury was accompanied by a decrease in arterial pO₂ (p<0.05, table S1) and required higher respiratory frequencies and peak airway pressure to maintain normo–pH (p<0.05, table S1) compared to non–infected controls. Systemically, pneumonia caused modest inflammation, with increased plasma levels of IL–6, but not of TNF–α, IL–1β and CINC3 when compared to non–infected controls (p<0.05, figure S2). Pneumonia resulted in an increase in kidney wet weight and urine protein content (both p<0.05, table 1), indicating kidney vascular damage and permeability edema. Plasma levels of ALT, but not AST, were increased during pneumonia (p<0.05, table 1). No abnormalities were observed on histopathologic examination of the liver and kidney.
Hydrogen sulfide reduces organ injury in pneumosepsis

Figure 1: NaHS reduced lung injury parameters in pneumosepsis. The number of cells (A), neutrophil influx (B), total protein (C), interleukin (IL)–6 (E), TNF-α (F), IL–1β (G) and CINC3 (H) in animals infected with S. pneumoniae and healthy controls was determined in bronchoalveolar lavage fluid (BALF). The right lung lobes were used to determine lung wet weight (D). Mean ± SEM, (n=6 in the pneumonia group infused with NaHS and n=8 in the other experimental groups). *: p<0.05.
Table S1: Arterial blood gas analysis and respiratory parameters at baseline (t=0) and after 4 hours of NaHS or saline infusion in rats with pneumosepsis and in healthy controls.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pneumonia</th>
<th>Healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>NaHS</td>
</tr>
<tr>
<td><strong>Time (hour)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>T = 0</td>
<td>7.39±0.07</td>
</tr>
<tr>
<td></td>
<td>T = 4</td>
<td>7.35±0.07</td>
</tr>
<tr>
<td>PaCO₂ (kPa)</td>
<td>T = 0</td>
<td>5.2±0.5</td>
</tr>
<tr>
<td></td>
<td>T = 4</td>
<td>4.9±0.9</td>
</tr>
<tr>
<td>PaO₂ (kPa)</td>
<td>T = 0</td>
<td>29±5</td>
</tr>
<tr>
<td></td>
<td>T = 4</td>
<td>24±5*</td>
</tr>
<tr>
<td>HCO₃⁻ (mmol/L)</td>
<td>T = 0</td>
<td>23±2</td>
</tr>
<tr>
<td></td>
<td>T = 4</td>
<td>20±5</td>
</tr>
<tr>
<td>Base excess</td>
<td>T = 0</td>
<td>−1.3±3.1</td>
</tr>
<tr>
<td></td>
<td>T = 4</td>
<td>−4.9±5</td>
</tr>
<tr>
<td>Respiratory rate (breaths/min)</td>
<td>T = 0</td>
<td>45±0*</td>
</tr>
<tr>
<td></td>
<td>T = 4</td>
<td>50±5</td>
</tr>
<tr>
<td>Peak pressure (cmH₂O)</td>
<td>T = 0</td>
<td>18±0*</td>
</tr>
<tr>
<td></td>
<td>T = 4</td>
<td>18±0</td>
</tr>
</tbody>
</table>

Data are means ± SD. *: Pneumonia + saline vs. pneumonia + NaHS, # healthy + saline vs. healthy + NaHS, †: pneumonia vs. healthy, p<0.05.

Figure S1: NaHS did not reduce bacterial outgrowth. The number of colonizing forming units (CFU) in bronchoalveolar lavage fluid (BALF) (A), lung (B), liver (C) and spleen (D) homogenates in animals infected with S. pneumoniae were determined by 10 fold dilutions on blood agar plates (n=8 in pneumonia group and n=6 in the NaHS group). Horizontal line indicates the mean (log scale).
Hydrogen sulfide reduces organ injury in pneumosepsis

Table 1: Physiological parameters at baseline and after 4 hours and organ function markers of rats with pneumonia or healthy controls after 4 hours of NaHS infusion or saline.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pneumonia Time (Hour)</th>
<th>Saline</th>
<th>NaHS</th>
<th>Healthy Time (Hour)</th>
<th>Saline</th>
<th>NaHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, (mmHg)</td>
<td>T = 0</td>
<td>120±23</td>
<td>121±23</td>
<td>T = 0</td>
<td>127±20</td>
<td>140±16</td>
</tr>
<tr>
<td></td>
<td>T = 4</td>
<td>91±24</td>
<td>95±19</td>
<td>T = 4</td>
<td>106±20</td>
<td>101±23</td>
</tr>
<tr>
<td>Heart rate, (beats/min)</td>
<td>T = 0</td>
<td>313±61</td>
<td>304±34</td>
<td>T = 0</td>
<td>306±37</td>
<td>287±37</td>
</tr>
<tr>
<td></td>
<td>T = 4</td>
<td>298±26</td>
<td>143±28</td>
<td>T = 4</td>
<td>306±37</td>
<td>136±63*a</td>
</tr>
<tr>
<td>Body temperature, (°C)</td>
<td>T = 0</td>
<td>36.6±1.2</td>
<td>36.6±0.6</td>
<td>T = 0</td>
<td>37.1±0.8</td>
<td>36.6±0.4</td>
</tr>
<tr>
<td></td>
<td>T = 4</td>
<td>37.4±0.5</td>
<td>24.8±1.6</td>
<td>T = 4</td>
<td>36.7±1.3</td>
<td>26.0±1.4</td>
</tr>
<tr>
<td>AST, U/L</td>
<td></td>
<td>194±186</td>
<td>121±33</td>
<td></td>
<td>89±44</td>
<td>98±47</td>
</tr>
<tr>
<td>ALT, U/L</td>
<td></td>
<td>127±143</td>
<td>62±26</td>
<td></td>
<td>47±11</td>
<td>49±12</td>
</tr>
<tr>
<td>Creatinine, mmol/L</td>
<td></td>
<td>50±9</td>
<td>27±3</td>
<td></td>
<td>40±7</td>
<td>23±4</td>
</tr>
<tr>
<td>GFR, mL/min</td>
<td></td>
<td>1.4±0.4</td>
<td>5.2±2.8</td>
<td></td>
<td>2.0±1.6</td>
<td>2.4±1.4</td>
</tr>
<tr>
<td>Kidney wet weight, g</td>
<td></td>
<td>1.62±0.13</td>
<td>1.46±0.09</td>
<td></td>
<td>1.19±0.37</td>
<td>1.38±0.06</td>
</tr>
<tr>
<td>Urine protein, mg/mL</td>
<td></td>
<td>0.71±0.58</td>
<td>0.29±0.39</td>
<td></td>
<td>0.16±0.25</td>
<td>0.13±0.14</td>
</tr>
</tbody>
</table>

Data are means ± SD. *: Pneumonia + saline vs. pneumonia + NaHS, # healthy + saline vs. healthy + NaHS, †: pneumonia vs. healthy, p<0.05.

MAP: mean arterial pressure, AST: aspartate aminotransferase, ALT: alanine transaminase, GFR: glomerular filtration rate.

Pneumosepsis–induced inflammatory responses and organ injury were accompanied by oxidative damage, reflected in higher protein carbonyl levels in both organs (p<0.05, figure 3). Mitochondrial oxygen consumption was markedly decreased during pneumosepsis compared to non–infected controls (p<0.05, figure 3), with a concomitant decrease in ATP and ATP/ADP ratios (p<0.05, figure 3). VDAC expression on the other hand was increased during pneumosepsis, reflecting a low bio–energetic status and enhanced ATP demand (p<0.05, figure 4). Also α–tubulin was increased compared to non–infected controls (p<0.05, figure 4). Low bio–energetic status was associated with decreased PGC1–α expression, a metabolism regulator (10) with concomitant low expression of mitochondrial respiratory subunit NADH6 in the lung (p<0.05, figure 5) as compared to non infected controls. Expressions of other subunits were not affected.
Figure 2: NaHS reduced histopathologic abnormalities and interstitial inflammation in pneumosepsis. Total histopathology score (A) and interstitial inflammation (B) with representative photographs of hematoxylin and eosin stained lung tissue sections (magnification x 20) of animals infected with *S. pneumoniae* infused with saline (C) or NaHS (D) and healthy controls (E+F). (n=6 in the pneumonia group infused with NaHS and n=8 in the other experimental groups). Mean ± SEM, *:p<0.05. Inserts represents interstitial inflammation.

**NaHS did not alter bacterial outgrowth of S. pneumoniae**

NaHS did not influence bacterial outgrowth in BALF and lung homogenates compared to saline controls, nor did it influence bacterial outgrowth in distant organs (figure S1). NaHS tended to decrease the number of positive blood cultures (38% vs. 13% in saline controls, p=0.13).
Hydrogen sulfide reduces organ injury in pneumosepsis

**Figure 3:** NaHS reduces oxidative damage and improved mitochondrial respiration and ATP availability in pneumosepsis.

Lung (A) and liver (B) protein carbonyls levels, with mitochondrial O2 consumption during oxidative phosphorylation of glutamate and malate (C+D) and concentrations of ATP (E) and ATP/ADP ratio (F) in animal infected with *S. pneumoniae* and healthy controls infused with saline or NaHS. Mitochondrial O2 consumption was recorded during state 3 and state 4 respirations in liver. Mean ± SEM, (n=6 in the pneumonia group infused with NaHS and n=8 in the other experimental groups). *: p<0.05.

**NaHS reduced heart rate, body temperature and improved gas exchange**

In line with our previous experiments (18), infusion of NaHS reduced heart rate and body temperature compared to saline controls, both in pneumonia and in healthy rats, without an effect on mean arterial pressure (all p<0.05, table 1). NaHS allowed for a reduction in respiratory rates compared to saline controls while maintaining normo–pH, in both infected and non–infected animals, with 20% and 26% respectively (p<0.05, table S1). NaHS prevented a fall in arterial pO2 in pneumonia and increased oxygenation in the healthy animals (p<0.05, table S1), but did not influence peak airway pressure.
NaHS reduced local and systemic inflammation and ameliorated kidney function in pneumosepsis

NaHS reduced BALF protein concentration compared to saline controls (p<0.05, figure 1), but did not reduce pulmonary edema. Also, NaHS reduced pulmonary cell counts and neutrophils influx as well as BALF levels of IL–6 and TNF–α (all p<0.05, figure 1). NaHS non–significantly decreased BALF levels of IL–1β and CINC3 compared to saline controls (p=0.08 and 0.18 respectively, figure 1). Inhibition of inflammation by NaHS was also reflected in the lung histopathology score, in particular interstitial inflammation (p<0.05, figure 2). NaHS did not affect AF concentration (48.5±10.4 vs. 29.1±3.7 U/L) compared to saline controls. NaHS decreased systemic levels of IL–6 (p<0.05, figure S2, supplemental data), but levels of TNF–α, IL–1β and CINC3 were not altered. NaHS infusion reduced kidney wet weight and protein loss in the urine (both p<0.05, table 1), together with an improved glomerular filtration rate and reduced plasma creatinine levels compared to saline controls (p<0.05, table 1), while neither cumulative fluid infusion nor diuresis differed. Liver enzymes were reduced by NaHS, which did not reach statistical significance due to a wide range (p=ns, table 1).

NaHS prevented a fall in bio–energetic status by maintaining mitochondrial respiration and by upregulation of mitochondrial biogenesis

Infusion of NaHS prevented the observed increase in levels of protein carbonyls (p<0.05, figure 3) in pneumonia compared to non–infected controls. In line with reduced oxidative stress, NaHS prevented the fall in active oxidative phosphorylation observed during pneumonia (p<0.05, figure 3), together with an increase in ATP concentration and ATP/ADP ratio (p<0.05, figure 3). Infusion of NaHS had no effect on the expression of VDAC, but resulted in upregulation of α–tubulin and the fraction of phosphorylated PKCε as regulators of VDAC in liver mitochondria (p<0.05, figure 4). In the lung, the effect of NaHS on regulators of VDAC was not observed. We found that infusion of NaHS reduced levels of COX1 and tended to reduce levels of NADH1 in BALF (p<0.05, figure S3). Circulating levels of mtDNA were decreased in a non–significant fashion by NaHS.
Hydrogen sulfide reduces organ injury in pneumosepsis

Figure S2: NaHS reduced systemic levels of interleukin (IL)–6 in pneumonia. The concentrations of IL–6 (A), tumor necrosis factor (TNF)–α (B), IL–1β (C) and CINC3 (D) in plasma of animals infected with S. pneumoniae and healthy controls infused with saline or H2S. (n=6 in the pneumonia group infused with H2S and n=8 in the other experimental groups). Mean ± SEM. *: p<0.05.

Besides improved mitochondrial function, NaHS infusion increased expression of liver PGC–1α, which is the main mitochondrial transcription factor (p<0.05, figure 5). NaHS also upregulated PGC1–α in healthy controls, indicating a ubiquitous effect of NaHS. In line with these results, hepatic NADH1 and NADH6 expression, which are subunits of mitochondrial respiratory complexes, increased after NaHS infusion (p<0.05, figure 5), albeit in a non–significant manner for NADH6. Taken together, these results may point to an enhanced mitochondrial biogenesis. Of note, we again found differences between the lung and liver. Upregulation of factors involved in mitochondrial biogenesis was noted in the liver, but not in the lung.
Figure 4: NaHS increased protein level of α–tubulin and protein kinase C (PKC)–ε as regulators of voltage dependent anion channel (VDAC) in pneumosepsis. Protein levels of VDAC (A), α–tubulin (B) and PKC–ε (C) on two separate immunoblots in liver freeze clamp biopsies of animals infected with *S. pneumoniae* or healthy controls infused with saline or NaHS. Data represent mean ± SEM from 6 animals per group. VDAC and α–tubulin are given as a ratio of prohibitin (PHB) expression. Phosphorylated (p) PKC–ε is given as a ratio of total (t) PKC–ε. Data as mean ± SEM, *: p<0.05.
Figure S3: NaHS maintained mitochondrial integrity reflected by low levels of mitochondrial DNA in bronchoalveolar lavage fluids (BALF). The expression of COX1 (A+B), NADH1 (C+D) and NADH6 (E+F) at mRNA level in plasma and in BALF in animals infected with *S. pneumoniae* or healthy controls infused with saline or H2S. Data represent mean ± SEM from n=6 in the pneumonia group infused with NaHS and n=8 in the other experimental groups.
Figure 5: NaHS increased mitochondrial biogenesis by up regulation of expression of PGC–1α and mitochondrial respiratory subunits in the liver. The expression of peroxisome proliferator–activated receptor gamma (PGC)–1α (A+B), COX1 (C+D), NADH1 (E+F) and NADH6 (G+H) at mRNA level in lung and liver in animals infected with *S. pneumoniae* or healthy controls infused with saline or NaHS. Data represent mean ± SEM from n=6 in the pneumonia group infused with NaHS and n=8 in the other experimental groups. Data as mean ± SEM, *: p<0.05.
Discussion

In this model of pneumococcal pneumosepsis, infusion of H$_2$S–donor NaHS reduced organ damage, associated with a reduction in oxidative damage as well as an increase in mitochondrial biogenesis, resulting in a reversal of inhibition of mitochondrial oxygen consumption and increased ATP bio–availability.

NaHS reduced lung injury by inhibiting inflammatory processes, in accordance with previous findings in non–infectious models of acute lung injury (18;21). NaHS reduced pulmonary neutrophil influx and protein leakage in the alveolar compartment during pneumonia. Preservation of the endothelial barrier may have been a mechanism of the observed decrease in distant organ injury in this study, as biomarkers of endothelial damage have been found in blood of patients with pneumonia, contributing to multiple organ failure (22).

Acute kidney injury is a common complication of pneumosepsis, with an important impact on outcome (23). In our model, pneumonia resulted in edema of the kidney, with urinary protein loss and deterioration of kidney function. Infusion of NaHS reversed the fall in the glomerular filtration rate and kidney function both in pneumonia as well as in healthy animals, suggesting that NaHS improved perfusion of the kidney. Decrease of protein leakage may presumably be due to anti–inflammatory effects of NaHS, thereby preserving the endothelial barrier in the glomeruli, comparable to effects in the pulmonary compartment.

NaHS tended to decrease liver cell damage, as reflected in decreased levels of both AST and ALT. We presume that significance was not reached due to a large variation in the levels of the liver enzymes, which would possibly be overcome with larger group size.

Under normal conditions, the electron transport chain complexes in the inner mitochondrial membrane are involved in formation of ATP. In sepsis, complex I and II are susceptible to damage, (24) which may lead to decreased ATP concentrations at cellular levels. In accordance, we found that in pneumonia, mitochondrial respiration was decreased when challenged with substrate for complex I, accompanied by reduced ATP and ATP/ADP ratios.

We hypothesize that low ATP levels in combination with a high expression of VDAC and impaired oxygen consumption during pneumonia may suggest an increased need for ATP in the presence of mitochondrial damage, presumably due to increased levels of pro–inflammatory cytokines and reactive oxygen species, as found in this study.

In sepsis, reactive oxygen species not only damage mitochondrial respiratory complexes, but also other vital proteins (25), thereby forming protein carbonyls (26). We found a reduction of protein carbonyls after NaHS infusion, which may have contributed to a reversal of the fall in mitochondrial respiration, together with increased ATP availability. This is in line with a previous study showing that NaHS reduced lung injury by improving the antioxidant glutathione pool inside the mitochondria (27). With respect to the mechanism of improved mitochondrial respiration and ATP bio availability, we found that NaHS increased the expression of proteins in the mitochondrial fraction which induce closure of VDAC, including
α–tubulin (12) and PKCε (28). VDAC is considered an important regulator of mitochondrial respiration (29). There may be several explanations for the apparent discrepancy between up–regulation of enzymes regulating closure of VDAC on the one hand and increased ATP and mitochondrial oxygen consumption on the other. NaHS has previously been found to induce a shift in substrate utilization towards increased glucose oxidation (14). Alternatively, closure of VDAC may induce suppression of ATP hydrolysis (30).

Taken together, we suggest that NaHS preserves mitochondrial oxidative phosphorylation during sepsis by increasing expression of mitochondrial α–tubulin and PKCε, thereby closing VDAC and stabilizing the mitochondrial membrane (31). Another indication that mitochondrial damage was limited by NaHS is the finding that levels of mtDNA in the bronchoalveolar lavage fluid were reduced, suggesting less leakage of mitochondria (32). As circulating or shed mtDNA invokes an inflammatory response in sepsis and trauma (32), a reduction in damaged mitochondria may have contributed to the protective effect of NaHS in this study. Although systemic mtDNA levels were non–significantly reduced after NaHS, we found a trend in decrease for all subunits, suggestive of a lack of power to demonstrate a significant effect.

We also found evidence for an improved mitochondrial biogenesis after NaHS infusion, as concluded from the upregulated expression of PGC1–α and transcription factors coding for respiratory complexes in pneumonia. Similarly to the effects of NaHS on mitochondria, other gas molecules like carbon monoxide and nitric oxide are able to enhance mitochondrial biogenesis (33).

The effects of NaHS on regulators of mitochondrial function were evident in the liver, but not in the lung. Of note, the lungs in this pneumonia model were severely damaged, which may underlie a lack of mitochondrial biogenesis. Also, the liver is a sentinel organ in regulating metabolism, whereas the lung is not. Our findings accord with experiments using carbon monoxide and nitric oxide, which also block cytochrome c oxidase, in which effects on liver mitochondria were apparent (33;34). Nevertheless, as we did not measure regulators of mitochondrial respiration in other organs, in particular in the kidney, the association between preserved organ function and improved mitochondrial function by NaHS is limited to the liver.

We previously reported that NaHS reversibly reduced metabolism in anesthetized rats (18), akin to the induction of a suspended animation like–state (17). It has been stated that reducing metabolism with concomitant hypothermia may render the host more susceptible to infection (35;36), although infection risk differs among studies (35–37). We did not find enhanced dissemination of bacteria during the hypothermic period. In contrast, there was a tendency to local containment of bacteria. This may result from the improved endothelial barrier function by NaHS, thereby inhibiting dissemination. However, an important question remains whether bacteria restart replicating after cessation of NaHS.
It may be argued that observed protective effects are attributable to hypothermia induced by NaHS. Although mild hypothermia reduces inflammation (38–40), detrimental effects of deep hypothermia have been reported in experimental settings (41;42) and in humans (43). In accordance, induction of deep hypothermia that paralleled effects of NaHS either worsened lung inflammation or had no effect in a rat model of ventilator induced lung injury (18;44) or shock (45;46). Thereby, we hypothesize that reducing metabolism by inhibiting mitochondrial respiration by NaHS while not allowing reducing body temperature may be toxic.

A limitation of our model of severe pneumosepsis is that survival studies are not possible. Thereby, whether the observed protective effect of NaHS on organ failure translates into improved outcome cannot be dissected from our studies. Another limitation may be that mitochondrial respiration was measured at 37°C, which does not represent the in vivo situation in NaHS infused animals. However, as the technique was the same in all groups, limitations pertaining to the measurement can not explain differences between NaHS and controls. Finally, we did not measure H₂S concentrations in tissue. However, NaHS infusion may be a more feasible treatment strategy then H₂S gas administration. Therefore, we consider results of this study relevant for future studies evaluating the use of NaHS in sepsis.

**Conclusion**

In this model of severe pneumosepsis, NaHS reduced local and distant organ injury, associated with maintained mitochondrial oxidative phosphorylation and improved mitochondrial biogenesis. Interventions aimed at stabilizing bio–energetic status may be a therapeutic approach to reduce organ failure in pneumosepsis.
References


Hydrogen sulfide reduces organ injury in pneumosepsis


Supplemental data

Methods and materials

Mechanical ventilation

Ventilator settings were determined in pilot experiments aiming at normo–pH (7.35–7.45) and tidal volumes of ~7.5 ml/kg (1). Rats with pneumonia were ventilated in a pressure controlled mode with 14 cmH₂O peak inspiratory pressure (PIP), 5 cm H₂O positive end expiratory pressure (PEEP). Non–infected rats were ventilated with 12 cmH₂O PIP and 5 cmH₂O PEEP. FiO₂ was set at 50%, inspiration to expiration ratio at 1:2, recruitment maneuvers were applied every 60 minutes by increasing PIP to 25 cmH₂O during 5 breaths. In pneumonia respiratory rate at baseline was 45 breaths/min and in the healthy controls 35 breaths/min. Respiratory rates were adjusted to maintain normo–pH in all groups.

Assays in BALF, plasma and tissue

Freezeclump and mitochondrial isolation

Part of the liver was removed by modified freeze clamping and placed in liquid nitrogen for ATP and adenosine diphosphate (ADP) measurements and part was placed in isolation buffer. To minimize ATP degradation and for mitochondrial viability, these steps were performed before exsanguination. For mitochondrial isolation, part of the liver was minced in isolation buffer (200 mmol/L mannitol, 50 mmol/L sucrose, 5 mmol/L KH₂PO₄, 5 mmol/L 3–(n–morpholino) propanesulfonic acid (MOPS), 1 mmol/L Ethylene glycol–bis(2–aminoethyl ether)–N,N,N′,N′–tetraacetic acid (EGTA), 0.1% bovine serum albumin), homogenized and centrifuged at 3220 x g for 10 min. Supernatant was removed, and the pellet was resuspended in 25 mL isolation buffer and centrifuged at 800 x g for 10 min. The supernatant was centrifuged at 3220 x g for 10 min. The final pellet was suspended in isolation buffer. Protein content was determined by the Bradford method (Bio–Rad, München, Germany). The isolation steps were conducted at 4°C.

Mitochondrial oxygen consumption

Mitochondrial oxygen consumption was measured polarographically at 37°C using a respiratory system (System S 200A, Strathkelvin Instruments, Glasgow, Scotland). Mitochondria (2 mg protein/ml) were suspended in respiration buffer (130 mmol/L KCl, 5 mmol/L K₂HPO₄, 20 mmol/L MOPS, 2.5 mmol/L EGTA, 1 μmol/L Na₄P₂O₇, 0.1% BSA, pH adjusted). Shortly after a stable signal was reached, mitochondrial respiration was initiated using glutamate (20mM, Aldrich, Steinheim, Germany) with malate (20mM, Aldrich, Steinheim, Germany). Exactly 60 s later, state 3 respiration was initiated by 200 μmol/L ADP injected into the respiration chamber. Respiration rates were recorded under state 3 conditions for approximately 4 minutes or after complete phosphorylation of ADP to ATP (state 4 respiration).
ATP and ADP determination

For nucleotide extraction, approximately 40–100 mg frozen tissue was grinded in liquid nitrogen using a ceramic mortar. Right before the liquid nitrogen has evaporated, the semi–viscous tissue powder was transferred to an eppendorf tube containing 200 µL of 0.4 M HClO₄ and placed on ice for 10 minutes. After centrifugation (10,000 x g for 10 minutes at 4°C), 150 µL of the supernatant was transferred to an empty Eppendorf tube and neutralized to pH 7.5–8.5 with 5 M K₂CO₃. The remainder of the supernatant was removed and 1000 µL of 0.2 M NaOH was added to the pelleted cell fragments, which was used for protein determination (BCA assay, Pierce, Etten–Leur, the Netherlands). The supernatants containing the nucleotides, including ATP and ADP, were stored at –80 until analysis by high performance liquid chromatography (HPLC).

Nucleotide analysis by HPLC

Nucleotide profiles were determined by ion–exchange HPLC, using a Whatman Partisphere SAX 4.6 × 125 mm column (5–µm particles) in combination with a Whatman 10 × 2.5 mm AX guard column (Clifton, NJ). The buffers used were 9 mM NH₄H₂PO₄, pH 3.5 (buffer A) and 325 mM NH₄H₂PO₄, 500 mM KCl, pH 4.4 (buffer B). Nucleotides were eluted with a gradient from 100% buffer A to 90% buffer B in a total run time of 60 min, at a flow rate of 1mL/min (2). The concentration of ATP and ADP were corrected for protein concentrations.

Mitochondrial fraction

Part of the liver freeze clamp biopsy and snapfrozen lung part was crushed in a pre–cooled mortar and quickly carried over to lysis buffer (Tris base, EGTA, NaF and Na₃VO₄ (as phosphatase inhibitors) containing protease inhibitor mix (aprotinin, leupeptin and pepstatin) and DTT and homogenized on ice. The liver homogenates were centrifugated at 1000 x g, 4°C for 10 min. To obtain the mitochondrial fraction, the supernatant was centrifuged at 10,000 x g, 4°C for 15 min. Again, the supernatant was centrifuged at 16,000 x g, 4°C for 15 min to obtain the cytosolic fraction.

Western Blot analysis

Total protein concentration was determined by the Lowry method. Subsequently, proteins were separated on a 4%–12% gradient gel (Criterion™ XT precast gel, Bio–Rad, the Netherlands) and transferred to a polyvinylidene fluoride membrane (Immobilon FL, Millipore, the Netherlands) by tank blotting. To prevent unspecific binding of the antibody, membranes were incubated with Odyssey Blocking Buffer (LI–COR, Westburg, Leusden, the Netherlands) with 0.1 % Tween (TBS–T) for 1 hour. Subsequently, the membrane was incubated over night at 4°C with the respective primary antibody anti–α–tubulin (Sigma Aldrich, St. Louis, MO), anti–VDAC (Calbiochem, Darmstadt, Germany), anti–phosphor PKCe,
anti–total PKCe (both Biomed, Huisen, the Netherlands) or anti–prohibitin 1 (PHB) (Cell Signal, Danvers, United States) diluted in 0.1% Tween Odyssey buffer. After washing in fresh, cold TBS–T 3x 5 min, the membrane was subjected to the appropriate secondary antibody for 1 hour at room temperature. Immuno–reactive bands on the membrane were visualized by the two channel laser system of the Odyssey system. The blots were quantified using the Odysseis IR Manager®. Equal loading of the protein to the SDS Page gel was ensured by Coomassie blue staining (Bio–Rad, Veenendaal, the Netherlands) and Ponceau staining (Sigma Aldrich).

**Lung, liver, plasma and BALF mRNA isolation**

Total RNA was extracted with Tripure (Roche diagnostics BV, Almere, Netherlands) from approximately 30 mg lung and liver tissue according to manufacturers’ instruction. Total DNA was isolated in 200µL plasma and BALF with Qiamp DNA kit (Qiagen, Venlo, Netherlands). RNA and DNA samples were quantified by spectrophotometry and stored at –80. RNA was converted to cDNA by using oligo dT as primers. Rat, COX1 (forward: 5’ATGACCCACCAATCACATGC3’ reverse: 5’ATCACATGGCTAGGCCGAG3’), NADH1 (forward: 5’ATACCCATGGGCAACCTCCT3’ reverse: 5’GGGCCTTTGCTAGTTGTAT3’), NADH6 (forward: 5’CTCACATGACAAAAACTAGCCCCCA3’ reverse 5’TCCACCTCAACTGCTGATGA3’), and PGC–1α (forward: 5’CAGAACCATGCAAACCACAC3’ reverse: 5’TGTGGGCTTTTGGTGTGAC3’) was analyzed by reverse–transcription–polymerase chain reactions (RT–PCRs) using lightCycler®SYBR green I master mix (Roche, Mijdrecht, the Netherlands) and measured in a LightCycler 480 (Roche) apparatus using the following conditions: 5 min 95°C hot–start, followed by 40 cycles of amplification (95°C for 10 seconds, 60°C for 5 seconds, 72°C for 15 seconds). Gene expression is presented as a ratio of the mean expression of the housekeeping genes HRPT (forward: 5’CCATCTTCCAGGAGGAGAT3’, reverse: 5’CTCCATGTTGGTGAAAGACG3’) and GAPDH (forward: 5’CCAGACAACTTTGTTTGGGAG3’, reverse: 5’GCTTTTCCACTTTCCGCTGAT3’).
Supplemental references
