Original Article

Protective effects of exogenous sodium hydrosulfide on the structure and function of myocardial mitochondria of mice with sepsis

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Abstract: Objective: This study aimed to investigate the protective effects of exogenous sodium hydrosulfide (NaHS) on the structure and function of myocardial mitochondria and the survival rate of mice with sepsis and the potential mechanism. Methods: 70 male C57BL/6 mice were randomly assigned into 7 groups: normal control group, sham group, sepsis group, sepsis + 25 μmol/L NaHS group, sepsis + 50 μmol/L NaHS group, sepsis + 100 μmol/L NaHS group and sepsis + 200 μmol/L NaHS group. Sepsis was induced by cecal ligation and perforation (CLP). NaHS was intraperitoneally injected. At 12 h after surgery, mice were sacrificed and the heart was harvested for the detection of microstructure, the contents of ATP, ROS, MDA and GSH, and mRNA expression of PGC-1α and Nrf2 mRNA. Another 50 mice were randomly divided into 5 groups: sepsis group, sepsis + 25 μmol/L NaHS group, sepsis + 50 μmol/L NaHS group, sepsis + 100 μmol/L NaHS group, and sepsis + 200 μmol/L NaHS group. Results: In sepsis group, the structure of myocardial mitochondria was significantly damaged, ATP reduced, ROS, and MDA increased, GSH reduced, and mRNA expression of PGC-1α and Nrf2 increased. When compared with sepsis group, the mitochondrial structure was improved, ATP increased, ROS and MDA reduced, GSH increased, and mRNA expression of PGC-1α and Nrf2 further increased after NaHS treatment, but these changes were NaHS concentration dependent. PGC-1α mRNA expression was positively related to Nrf2 mRNA. MDA content was negatively associated with PGC-1α and Nrf2 mRNA expression. ATP and GSH contents were positively related to PGC-1α and Nrf2 mRNA expression. The 48 hours survival rate after modeling of mice in NaHS intervention group was higher than that in CLP group, and which was the highest in 100 μmol/L group. Conclusion: Exogenous NaHS is able to increase myocardial GSH content and PGC-1α and Nrf2 mRNA expression to combat with oxidative stress in sepsis, which is helpful for the improvement of myocardial mitochondrial structure and function and the survival rate of sepsis mice. 100 μmol/L is the optimum experimental concentration in this study.

Keywords: Sepsis, mitochondria, GSH, PGC-1α, Nrf2

Introduction

Sepsis is a serious systemic inflammatory response syndrome caused by External pathogenic microbial infection [1]. It may rapidly progress into serious sepsis, septic shock and multiple organ dysfunction syndrome (MODS) and has been a major infection related disease with high mortality and morbidity [2]. To date, studies [3] have shown that mitochondrial injury and dysfunction are one of important molecular mechanisms underlying the pathogenesis of sepsis. Mitochondrion is an important energy metabolism center and a major site of reactive oxygen species (ROS) production [4]. In case of sepsis, excess production of ROS may cause oxidative stress, leading to mitochondrial injury [3]. ROS may affect the activity of mitochondrial respiratory chain complex to interrupt its oxidative phosphorylation, which then reduce the ATP production in cells. In addition, ROS may also alter the mitochondrial membrane permeability and induce the release of Ca²⁺ and cytotoxic factors (such as Cyt C), resulting in cytotoxicity. ROS can interrupt the transcription and translation of mtDNA and inhibit the synthesis of mtDNA encoding proteins, affecting the normal expression of mitochondrial proteins. ROS may direct attack the biomacromolecules in cells (such as proteins, lipids and nucleic acids),
Exogenous sodium hydrosulfide affects myocardial mitochondria

inducing cell oxidative injury. These injuries may further increase the production of ROS, resulting in a vicious cycle [5, 6].

Peroxisome proliferator activated receptor gamma co-activator 1α (PGC-1α) is a transcriptional coactivator and has diverse biological activities similar to transcriptional factors. It may facilitate the mitochondrial oxidative expression and elevate mitochondrial oxidation function [7]. PGC-1α is potent to induce the expression of nuclear related factor 2 (Nrf2) and both factors then act simultaneously to regulate the mitochondrial biosynthesis [8]. There is evidence showing that exogenous sodium hydrosulfide (NaHS) as a donor of hydrogen sulfide (H₂S) is protective on mitochondrial function in pathological condition [9].

This study was undertaken to investigate the protective effects of exogenous NaHS on the mitochondrial structure and function of myocardial cells of mice with sepsis and explore the influence of exogenous NaHS on the myocardial MDA and GSH content as well as PGC-1α and Nrf2 mRNA expression in the heart, which may provide theoretical evidence on the therapeutic effects of NaHS on sepsis.

Materials and methods

Animals and CLP model

C57BL/6 male specific pathogen free (SPF) mice aged 6-8 weeks and weighing 20-30 g were purchased from Beijing HFK Bioscience Co. Ltd. Seventy mice were randomly divided into 7 groups (n=10 per group): Group A (normal control), Group B (sham-operation), Group C (sepsis was induced by cecal ligation and puncture [CLP]), Group D (sepsis was treated with 25 μmol/L NaHS [i.p.]), Group E (sepsis was treated with 50 μmol/L NaHS [i.p.]), Group F (sepsis was treated with 100 μmol/L NaHS [i.p.]), and Group G (sepsis was treated with 200 μmol/L NaHS [i.p.]). Mice were given ad libitum access to food and water. Mice were allowed to acclimate to the environment for one week before experiment. Sepsis was induced by CLP as previously described [10]. An intraperitoneal injection of 7% chloral hydrate solution (0.5 ml/100 g) was administered for anesthesia and then the abdominal skin was prepared with 75% alcohol disinfection. After a midline incision was made, the cecum was explored and exposed. Ligation was conducted at about 1 cm to the end of cecum to cause sepsis. After replacement of the cecum into the abdominal cavity, the wound was closed, and NaHS (Sigma) was intraperitoneally administered. After operation, normal saline (0.2 ml/mouse) was subcutaneously administered. After 12 h, mice were sacrificed by cervical dislocation after anesthesia and the myocardial tissues were collected for subsequent experiments. Another 50 mice were randomly divided into 5 groups: sepsis group, sepsis + 25 μmol/L NaHS group, sepsis + 50 μmol/L NaHS group, sepsis + 100 μmol/L NaHS group, and sepsis + 200 μmol/L NaHS group. Then the 48 hours survival rate after modeling of mice were compared among groups. All procedures were in accordance with the United Kingdom Animal (Scientific Procedures) Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

TEM

The myocardial tissue was cut into blocks (about 1 mm³). After fixation in glutaraldehyde and osmium chloride, tissues were dehydrated in gradient acetone solution, embedded, cut into sections and then stained with compound dye (0.25% sodium borate: 0.25% basic fuchsin =1:1). After cell image observation by the microscope, the sections were cut into ultrathin sections. The ultrathin sections were mounted on a copper wire mesh attached with a film prepared with 0.45% Fonnvar solution. After double stained at room temperature by uranyl acetate staining fluid and lead staining fluid, the sample was dried by filter paper. Then TEM was performed, and representative photographs were captured.

Isolation and detection of myocardial mitochondria

Myocardial mitochondria were isolated from each group. Briefly, the heart was quickly excised and washed in a buffer (pH 7.4) containing 250 mM sucrose, 10 mM Tris and 1 mM EGTA at 4°C. Then, the myocardial tissues were cut into small blocks and homogenized, followed by centrifugation at 700 r/min for 10 min. The supernatant was collected and centrifuged at 1000 r/min for 15 min. Subsequently, the supernatant was removed and myocardial
Exogenous sodium hydrosulfide affects myocardial mitochondria

Table 1. Primers used for fluorescent quantitative PCR

<table>
<thead>
<tr>
<th></th>
<th>Sequence (5’-3’)</th>
<th>Length (bp)</th>
</tr>
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<tbody>
<tr>
<td>Nrf2 F</td>
<td>CTTCCATTACGGAGACCCAC</td>
<td>175</td>
</tr>
<tr>
<td>Nrf2 R</td>
<td>GATTCACGCATAGGAGCACTG</td>
<td></td>
</tr>
<tr>
<td>PGC-1α F</td>
<td>TGCCACGGACCCCTATTC</td>
<td>210</td>
</tr>
<tr>
<td>PGC-1α R</td>
<td>GAGGATCTACTGCTGGGGAC</td>
<td></td>
</tr>
<tr>
<td>Actin F</td>
<td>GAGACCTTCAACACCCAGC</td>
<td>263</td>
</tr>
<tr>
<td>Actin R</td>
<td>ATGTCACGCACGATTCCC</td>
<td></td>
</tr>
</tbody>
</table>

Mitochondria were obtained. All isolated mitochondria were kept on ice and used within 3 h. Some myocardial tissues were taken from the left ventricle. Thereafter, the protein concentration was determined. Then, the protein concentration of each sample was adjusted to 0.5 mg/ml with mitochondria determination solution. The ATP content of myocardial mitochondria was detected by an ATP kit (BioVision).

Oxidative stress and antioxidant detection

Intracellular ROS were detected by using a ROS assay kit with an oxidation-sensitive fluorescent probe (DCFH-DA) in a spectrofluorometer (excitation 500 nm, emission 520 nm). The malondialdehyde (MDA) content was detected with thiobarbituric acid (TBA) method. The reduced glutathione (GSH) content was detected with dithionitrobenzoic acid (DTNB) method.

Detection PGC-1α and Nrf2 mRNA expression

In brief, 1 ml of TRIzol reagent (Qiagen) was added to 50-100 mg of myocardial tissues, followed by homogenization. The supernatant was collected as total RNA. Random primers were used for the reverse transcription of total RNA by SYBR green I fluorescent quantitative PCR (Eppendorf). The mRNA expression of PGC-1α and Nrf2 was detected with following primers (Table 1). PCR conditions were as follows: 94°C for 4 min, 94°C for 20 sec, 60°C for 30 sec and 72°C for 30 sec, a total of 35 cycles. Detection of each sample was performed in triplicate.

Statistical analyses

Statistical analysis was performed with the Statistical Product for Social Sciences (SPSS; version 20.0). Comparisons among the groups were done by one-way analysis of variance. Pearson’s correlation coefficient was used to describe the correlation between parameters. Comparison of survival rate was analyzed by survival curve and Kaplan-Meier log-rank method. A value of P<0.05 was considered statistically significant.

Results

Myocardial mitochondrial structure

In normal control group and sham group, the myocardial mitochondria showed regular shape, clear boundary, even matrix, and dense mitochondrial crest, and there were no evident swelling and vacuolar degeneration. The myocytes had complete cell membrane and clear intercellular boundary, filaments were regularly arranged, and fibrous structure in the interstitium did not increase significantly. In sepsis group, the number of myocardial mitochondria reduced, mitochondria showed irregular shape, obvious swelling and vacuolar degeneration were observed, and there was evident mitochondrial crest rupture in the swelling mitochondria. The filaments were irregularly arranged, and the fibrous structure increased significantly in the interstitium. There was also obvious fiber fracture and lysis in some parts of the myocardium. In sepsis + 25 μmol/L NaHS group, the myocardial mitochondrial morphology was similar to that in sepsis group. In other NaHS groups, the myocardial mitochondrial morphology was improved as compared to sepsis group, the number of mitochondria increased in a NaHS concentration dependent manner, mitochondrial shape became regular, mitochondrial swelling and vacuolar degeneration were attenuated, and the fibrous structure in the interstitium reduced as compared to sepsis group (Figure 1).

Myocardial mitochondrial ATP content

Our results showed the ATP content was comparable between normal control group and sham group (t=0.357, P=0.725). In sepsis group, myocardial ATP content reduced significantly as compared to sham group (t=65.173, P=0.000). After treatment with NaHS at different concentrations, myocardial ATP content increased markedly as compared to sepsis group (F=884.510, P=0.000) and the ATP con-
Exogenous sodium hydrosulfide affects myocardial mitochondria

Figure 1. Morphology of myocardial mitochondria in different groups (TEM 20000×). A: Normal control group; B: Sham group; C: Sepsis group; D: Sepsis + 25 μmol/L NaHS group; E: Sepsis + 50 μmol/L NaHS group; F: Sepsis + 100 μmol/L NaHS group; G: Sepsis + 200 μmol/L NaHS group.

Figure 2. Myocardial ATP content of each group (n=10 per group). Mice were submitted to CLP for sepsis induction, and then received intraperitoneal injection of NaHS at different concentrations. Mice were sacrificed 12 h later, and the myocardial tissue collected for detection of ATP content. *P<0.05 vs. sham group, **P<0.05 vs. sepsis group. Error bar: 95% confidence intervals.

Figure 3. The ROS level of each group (n=10 per group). The mice were submitted to CLP procedure for sepsis induction. Meanwhile, intraperitoneal injection of NaHS in corresponding concentration was administered. 12 h later, the mice were euthanized and myocardial tissue collected for subsequent analysis. The ROS levels were detected by spectrofluorometer. *P<0.05 vs. normal control group, *P<0.05 vs. sham-operation group, **P<0.05 vs. sepsis group. The error bars are indicative of the 95% confidence intervals.

Myocardial ROS content

The ROS levels in the sham-operation group and the sepsis groups had increased to various degrees compared with the normal control group. NaHS affected the ROS levels in a concentration-dependent manner, which only decreased in 100, 200 μmol/L NaHS groups (Figure 3).

Myocardial MDA content

In sham group and normal control group, the myocardial MDA content was similar (t=1.894, P=0.074). In sepsis group, the MDA content increased significantly as compared to sham group (t=13.875, P=0.000). After treatment with NaHS at different concentrations, the myocardial MDA content reduced dramatically as compared to sepsis group (F=141.235, P=0.000), but there was no significant difference between 100 and 200 μmol/L NaHS groups (Figure 4).
Exogenous sodium hydrosulfide affects myocardial mitochondria

Myocardial GSH content

The myocardial GSH content was comparable between sham group and normal control group (t=0.218, P=0.830). In sepsis group, the myocardial GSH content reduced markedly as compared to sham group (t=3.745, P=0.001). Intraperitoneal treatment with NaHS at different concentrations dramatically increased the myocardial GSH content as compared to sepsis group (F=40.984, P=0.005) (Figure 5).

mRNA expression of PGC-1α and Nrf2 in the myocardium

The mRNA expression of PGC-1α and Nrf2 in the myocardium was similar between normal control group and sham group (PGC-1: t=0.740, P=0.469; Nrf2: t=1.891, P=0.075). The mRNA expression of PGC-1α and Nrf2 in the myocardium of sepsis group increased significantly as compared to normal control group and sham group (PGC-1: F=84.426, P=0.000; Nrf2: F=142.774, P=0.00). After treatment with NaHS at different concentrations, the mRNA expression of PGC-1α and Nrf2 in the myocardium.
Exogenous sodium hydrosulfide affects myocardial mitochondria

In sepsis mice treated with NaHS, PGC-1α mRNA expression was positively related to Nrf2 mRNA expression (r=0.918, P=0.000). ATP content was positively related to PGC-1α and Nrf2 mRNA expression (PGC-1α: r=0.821, P=0.000; Nrf2: r=0.863, P=0.000). MDA content was negatively related to PGC-1α and Nrf2 mRNA expression (PGC-1α: r=-0.925, P=0.000; Nrf2: r=-0.887, P=0.000). GSH content was positively related to PGC-1α and Nrf2 mRNA expression (PGC-1α: r=0.843, P=0.000; Nrf2: r=0.822, P=0.000).

Comparison of the 48 hours survival rate of mice

The 48 hours survival rate after modeling of mice in NaHS intervention group were higher than that in CLP group and that in 100 μmol/L was the highest (Figure 7).

Discussion

The influence of sepsis on mitochondria is still controversial, and some studies report the increased, reduced and unchanged mitochondrial function in case of sepsis [11-13]. Mitochondria are an energy metabolism center and the major site where energy is produced for the cell metabolism. To date, some studies [3, 14] indicate that mitochondrial injury and dysfunction may cause abnormal production of ATP to affect the organ function, which is one of important molecular mechanism of sepsis. In the present study, results showed the structure of myocardial mitochondria was significantly damaged in sepsis, accompanied by the evident reduction in myocardial ATP, which supports the damage to the structure and function of myocardial mitochondria in case of sepsis.

Figure 7. The 48 hours survival rate after modeling of mice. At 48 hours after modeling, the survival rate of mice in sepsis was 40%, and 50% in sepsis + 25 μmol/L NaHS group, 70% in sepsis + 50 μmol/L NaHS group, 100% in sepsis + 100 μmol/L NaHS group, 80% in sepsis + 200 μmol/L NaHS group. NaHS could improve the survival rate of sepsis mice and 100 μmol/L was the best concentration.
Exogenous sodium hydrosulfide affects myocardial mitochondria

pro-inflammatory cytokine production, leading to the attenuation of inflammatory cascade in systemic inflammatory reaction and the improvement of pathophysiology of sepsis [17, 18]. However, in acute inflammation, the antioxidants are insufficient to scavenge ROS [19]. Our results showed GSH content in sepsis group reduced significantly as compared to sham group and normal control group, suggesting the compromised anti-oxidation and imbalance between oxidation and anti-oxidation, which leads to the deterioration of injury. Thus, to increase the anti-oxidants (such as GSH) and reconstruct the redox balance may be therapeutic for sepsis.

Nrf2 is an important transcriptional activator in the oxidative stress, acts a core factor in the oxidative reaction and is crucial for the maintenance of redox balance [20, 21]. Our results showed Nrf2 mRNA expression increased significantly in sepsis mice as compared to mice in sham group and normal control group. This indicates that the oxidative stress in case of sepsis may up-regulate Nrf2 mRNA expression as a response. The binding of Nrf2 to its negative regulatory factor Keap1 reduces, which may decrease the degradation of Nrf2 and increase its stability and activity, and then the activated Nrf2 enters the nucleus, leading to the up-regulated expression of genes related to anti-oxidation [22, 23]. PGC-1α is the first member of PGC-1 family and may be specifically expressed in tissues. That is, PGC-1α is mainly expressed in tissues with high energy requirement or rich in mitochondria (such as heart, skeletal muscle, kidney and liver). PGC-1α is a transcriptional coactivator. In recent years, study reports that PGC-1α gene deficiency may inhibit the expression of several anti-oxidation related genes [24]. Our results showed PGC-1α mRNA expression in sepsis mice was markedly higher than in normal control group and sham group and positively related to Nrf2 mRNA expression. This indicates that PGC-1α expression is up-regulated as a response to oxidative stress in case of sepsis, and its mRNA expression is closely related to the transcription of Nrf2. St-Pierre et al [24] found that there was ARE in the promoter of PGC-1α gene, and Nrf2 could bind to the ARE in the promoter of PGC-1α gene to regulate PGC-1α expression. PGC-1α acts together with Nrf2 to regulate the anti-oxidation, which plays an important role in maintaining the redox balance.

H₂S is an important endogenous biological signaling molecule and possesses anti-oxidative, anti-inflammatory and anti-apoptotic activities. It play crucial roles in the physiology and pathophysiology of cardiovascular system, digestive system and neurological system [25, 26]. NaHS is an exogenous donor of H₂S and may improve the mitochondrial function [9]. Our results showed NaHS at different concentrations was able to up-regulate PGC-1α and Nrf2 mRNA expression in a concentration dependent manner. Comparing with those in CLP group, MDA levels decreased in all NaHS intervention groups, but there was no significant difference between 100 and 200 μmol/L NaHS groups. ROS levels only decreased in 100 and 200 μmol/L NaHS groups. In addition, NaHS at different concentrations increased ATP and GSH contents. Moreover, MDA content was negatively related to PGC-1α and Nrf2 mRNA expression, and ATP and GSH contents were positively associated with PGC-1α and Nrf2 mRNA expression. These findings suggest that NaHS may up-regulate the mRNA expression of PGC-1α and Nrf2 in the myocardium of sepsis mice. PGC-1α and Nrf2 may act synergistically to increase GSH to combat with oxidative stress and reduce MDA, which improves mitochondrial function and increase ATP content. The 48 hours survival rate after modeling of mice in NaHS intervention group was higher than that in CLP group, which was the highest in the 100 μmol/L group.

Taken together, exogenous NaHS may increase myocardial PGC-1α and Nrf2 mRNA expression and GSH content in a dose dependent manner, which then regulates the antioxidative capability to combat with oxidative stress, improves the structure and function of mitochondria, and then improves the survival rate of sepsis mice. Thus, 100 μmol/L is the optimum experimental concentration in this study.

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Disclosure of conflict of interest

None.
Exogenous sodium hydrosulfide affects myocardial mitochondria

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Exogenous sodium hydrosulfide affects myocardial mitochondria

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