Methionine metabolism in an animal model of sepsis

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Abstract

Background: Sepsis is a disease with high incidence and lethality and is accompanied by profound metabolic disturbances. In mammalian methionine metabolism, S-adenosylmethionine (SAM) is produced, which is important in the synthesis of neurotransmitters and glutathione and as an anti-inflammatory agent. The degradation product and antagonist of SAM is S-adenosylhomocysteine (SAH). In this study, we investigated changes in methionine metabolism in a rodent model of sepsis.

Methods: Sepsis was induced in male Wistar rats (n=21) by intraperitoneal injection of bacterial lipopolysaccharide (10 mg/kg). Controls (n=18) received vehicle only. Blood was collected by cardiac puncture 24 h later. Puncture of the suboccipital fossa was performed to collect cerebrospinal fluid (CSF). Methionine metabolites were measured using stable isotope dilution tandem mass spectrometry. Plasma total homocysteine and cysteine were measured by HPLC using fluorescence detection. Glutathione was assayed using a modified enzymatic microtiter plate assay. Results: We observed significantly higher plasma levels of SAM (p<0.001) and SAM/SAH ratio (p=0.004) in septic animals. In CSF, there was also a trend for higher levels of SAM in septic animals (p=0.067). Oxidative stress was reflected by an increase in the ratio of oxidized/reduced glutathione in septic animals (p=0.001).

Conclusions: Sepsis is associated with an increased SAM/SAH ratio in plasma and CSF in rodents. This indicates an altered methylation potential during sepsis, which may be relevant for sepsis-associated impairment of transmethylation reactions, circulation and defense against oxidative stress. If verified in humans, such findings could lead to novel strategies for supportive treatment of sepsis, as methionine metabolism can easily be manipulated by dietary strategies.
Methionine metabolism in an animal model of sepsis

Alexander Semmler$^{1,2}$, Yvo Smulders$^3$, Eduard Struys$^4$, Desiree Smith$^3$, Susanna Moskau$^1$, Henk Blom$^3$, Michael Linnebank$^{1,2}$

$^1$University Hospital Zurich, Department Neurology, Switzerland
$^2$University Hospital Bonn, Department Neurology, Germany
$^3$VU University Medical Center, Department Internal Medicine and Institute for Cardiovascular Research-ICaR-VU, Amsterdam, The Netherlands
$^4$VU University Medical Centre, Metabolic Unit, Department of Clinical Chemistry, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands

Correspondence: Dr. Alexander Semmler, University Hospital Bonn, Sigmund-Freud-Str. 25, D-53125 Bonn, Germany. Phone: +49 228 287-15712. Fax: +49 228 287-15455. E-mail: alexander.semmler@ukb.uni-bonn.de

Running title: Methionine metabolism in sepsis
Abstract

**Background:** Sepsis is a disease with a high incidence and a high lethality. Sepsis is accompanied by profound metabolic disturbances. In mammalian methionine metabolism, S-adenosylmethionine (SAM) is produced, which is important for the synthesis, e.g., of neurotransmitters and glutathione, and SAM acts as an anti-inflammatory agent. Degradation product and antagonist of SAM is S-adenosylhomocysteine (SAH). This study investigated changes in this metabolism during sepsis in a rodent model.

**Methods:** Sepsis was induced in Male Wistar rats (n=21) by an intraperitoneal injection of bacterial lipopolysaccharide (LPS, 10mg/kg). Controls (n=18) received the vehicle only. 24 hours after injection 1ml blood was collected by cardiac puncture. Puncture of the suboccipital fossa was performed to collect cerebrospinal fluid (CSF). Methionine metabolites were measured by using stable isotope dilution tandem mass spectrometry. Plasma total homocysteine and cysteine were measured by HPLC using fluorescence detection. Glutathione was assayed by a modified enzymatic microtiter plate assay.

**Results:** We observed significantly higher plasma levels of SAM (p<0.001) and of the ratio of SAM/SAH (p=0.004) in septic animals. In CSF, septic animals also had higher levels of SAM for trend (p=0.067). Oxidative stress was reflected by an increase in the ratio of oxidized/reduced glutathione in septic animals (p=0.001).

**Conclusions:** Sepsis is associated with an increase in the SAM/SAH ratio in plasma and CSF in rodents. This indicates an altered methylation potential during sepsis, which may be relevant for sepsis-associated impairment of transmethylation reactions, circulation and defence against oxidative stress. If verified in humans such findings could lead to novel strategies of supportive treatment of sepsis, as methionine metabolism can easily be manipulated by dietary strategies.

Keywords: sepsis; homocysteine metabolism; transmethylation; glutathione; S-
adenosylmethionine; oxidative stress; DNA methylation

**Introduction**

Despite considerable efforts sepsis is still one of the leading causes of death in intensive care units (1, 2). Sepsis markedly alters metabolism, in particular amino acid and protein metabolism (3, 4). During transmethylation of methionine to homocysteine, methionine is transferred to S-adenosylmethionine (SAM; Fig1). SAM occupies a central position in the metabolism of all cells as an essential methyl donor to maintain normal methylation of DNA, RNA, proteins, phospholipids, histones and neurotransmitters as well as a multitude of small molecules necessary for normal cell function and viability. SAM can control the expression of multiple genes in a dose dependent manner by binding to riboswitches that control transcription and translation (5). In sepsis SAM inhibits the decrease of circulating immunomodulatory cells and the increase of the pro-inflammatory cytokine IL-1 (6). The degradation product of SAM is S-adenosylhomocysteine (SAH), which is reversibly hydrolyzed to homocysteine (Hcys). Hcys can be transsulfurated into cystathionine via cystathionine beta-synthase. Further, via cystathionine gamma-lyase, cystathionine is catalyzed to cysteine as a component of glutathione (GSX), which is important for the cellular redox system. As an alternative to transsulfuration, Hcys can be recycled by remethylation to methionine via methionine synthase. This requires vitamin B12 and 5-methyltetrahydrofolate (5-MTHF), which is synthesized by 5,10-methylenetetrahydrofolate reductase (MTHFR) from 5,10-methylenetetrahydrofolate (5,10-MTHF). In addition, the latter folate derivative is necessary for nucleic acid synthesis. Supraphysiological doses of vitamin B12 and vitamin B2, a cofactor of MTHFR, are beneficial in sepsis (7, 8). LPS is a major cell wall constituent of Gram-negative bacteria. The LPS-model of sepsis induction works through binding to the LPS-binding protein (LBP), endothelial activation through a receptor complex of Toll-like receptor 4
(TLR4), the LPS receptor molecule (CD14), and MD2, as well as early activation of nuclear factor-κ-B (NF-κB), finally resulting in the production of various pro-inflammatory mediators and possibly cell injury (9).

In view of the central importance of methionine metabolism for overall cellular viability and oxidative defence and the beneficial role of cofactors of important enzymes of methionine metabolism during sepsis, we aimed at investigating the effects of sepsis on essential components of methionine metabolism. We compared the ratios SAM/SAH, oxidized/reduced glutathione (GSSG/GSH) and Hcys and cysteine levels in LPS-treated and control rats. The ratio SAM/SAH is important because it is used as an indicator of the cellular methylation potential, as SAM and SAH are the substrate and product, respectively, of essential methyltransferase reactions, and SAH antagonises effectively SAM-dependent transmethylation reactions. The ratio GSSG/GSH is a common measure for the cellular redox potential, and the Hcys and cysteine levels are indicative of the transsulfuration activity of the methionine metabolism (10). As the involvement of the central nervous system is a common complication of sepsis and is associated with a higher mortality in sepsis patients (11, 12), we additionally measured SAM and SAH in the CSF.

Methods

Male Wistar rats (Charles River, Germany) weighing 250 – 300 g were housed in groups of four under standard conditions at a temperature of 22°C (±1°C) and a 12h light-dark cycle with free access to standard food pellets (Altromin, Soest, Germany) and tap water.

To initiate experimental sepsis, rats received LPS (0127:B8, E. coli (10mg/kg) dissolved in 1ml sodium chloride (0.9%) intraperitoneally (i.p.; n=22). Control animals received the vehicle (sodium chloride) alone (n=18). 24 hours after initiation of experimental sepsis animals were anesthetized with ketamine (75 mg/kg) and xylazine hydrochloride (10 mg/kg)
i.p. Puncture of the suboccipital fossa was performed to collect approximately 100μl of CSF. 1ml blood was collected by cardiac puncture and stored in EDTA-containing vials. All animals were sacrificed by decapitation. Septic and control animals were treated in parallel in groups of 10 each. The animal experiments were approved by the local government. To obtain plasma, the blood was centrifuged for 15 min at 4000g and 4ºC, immediately after collection. The supernatant was deproteinized by perchloric acid 10%. All aliquots were stored at -80ºC for 1-2 months before shipment on dry ice. Deproteinized plasma and CSF were used for simultaneous determination of SAM and SAH by using stable isotope dilution tandem mass spectrometry as previously published. Inter-assay coefficients of variation were 6.8% for SAM and 6.9% for SAH (13). Due to the limited amounts of available CSF, we restricted CSF analysis to the “key parameters” SAM and SAH. Plasma total homocysteine and cysteine were measured by HPLC using fluorescence detection. Inter-assay coefficients of variation for homocysteine and cysteine were 1.8 and 3.6%, respectively (14). Glutathione was assayed as total cellular glutathione, i.e. the sum of the reduced and oxidized forms by a modified enzymatic microtiter plate assay (15, 16). Briefly, pellets of EDTA blood were treated with sulfosalicylic acid prior to centrifugation and freezing of the supernatant. Total cellular glutathione content was determined after addition of dithiobisnitrobenzoic acid (DTNB) to aliquots of the supernatant at a final concentration of 10mM by absorbance changes at 405nm for 10 min. To determine oxidized glutathione, reduced glutathione was derivatized with 2.5% 2-vinylpyridine for 60 min prior to kinetic measurement. Protein content was determined using the Lowry method (17). Reduced glutathione was calculated from the difference of total glutathione and oxidized glutathione.

Statistics

Differences between groups were calculated with the two-sided t-test, and correlation analysis was performed using the Pearson’s correlation coefficient. A p-value of ≤0.05 was considered
Results

Experimental induction of sepsis led to typical symptoms such as piloerection, tachypnoea and social withdrawal. One rat of the sepsis group died during the observation period. All other rats survived LPS and vehicle treatment. Thus, groups of LPS-treated versus control animals included 21 and 18 rats, respectively.

The plasma and CSF results are listed in Table 1. In the LPS-treated rats, the plasma SAM/SAH-ratio was significantly higher (p=0.004) than in the control animals due to higher SAM levels (p<0.001). Comparable, but not significantly different results were obtained for the changes of SAM (p=0.067) and SAH levels in CSF. SAM in plasma and SAM in CSF correlated significantly both in septic animals (r = 0.460; p = 0.041) as well as in controls (r = 0.691; p = 0.006). There was no significant difference between Hcys and cysteine between both groups. The levels of total glutathione did not significantly differ, either. However, the relative amount of oxidized glutathione (GSSG) was significantly higher (p=0.022), and reduced glutathione (GSH) was significantly lower (p<0.001), and consecutively, the ratio oxidized/reduced glutathione was significantly higher (p=0.001) in septic animals.

In explorative analysis in septic animals we found a correlation between plasma Hcys and SAH levels (r = 0.601; p = 0.011), between Hcys plasma and total cellular glutathione blood levels (r = 0.633; p = 0.011), and, for trend, between SAM plasma and total cellular glutathione blood levels (r = 0.434; p = 0.064).

Discussion

Protein metabolism is markedly changed during sepsis, characterized by a generalized protein catabolism in the muscle accompanied by an enhanced elimination of amino acids from the intravascular space mainly to the liver (3, 18). Proteolysis might contribute to the observed
increase in SAM. SAM is the universal methyl donor for all methyltransferases. Sepsis-induced inhibition of these methyltransferases may lead to impaired utilisation of SAM and contribute to the rise in SAM-levels. Increased Hcys-remethylation to methionine and finally SAM during sepsis is unlikely, as oxidative stress leads to decreased activities of methionine synthase and betaine-homocysteine S-methyltransferase (19-21). In addition, we would expect Hcys levels to be lower in septic animals in the case of increased remethylation during sepsis. Also, high SAM levels down-regulate MTHFR, a key enzyme in Heys-remethylation, making it unlikely that remethylation is increased (22). Finally, the increase of SAM might be due to an enhanced synthesis of SAM from methionine during sepsis. The present data do not allow any conclusion on the underlying mechanism.

Because SAM and SAH are the substrate and product of essential methyltransferase reactions, and because SAH is an effective antagonist of transmethylation enzymes, the ratio of SAM/SAH is frequently used as an indicator of the cellular methylation potential (23, 24). The ratio of SAM/SAH of 6.55 found in the controls in the current study corresponds well to the SAM/SAH ratios found in humans in former studies under physiological conditions (24, 25). However, this ratio augments during sepsis indicating an increased methylation potential, which will influence numerous reactions involved in cellular metabolism, DNA methylation, membrane stability and neurotransmitter synthesis (26-28).

In the CSF of septic rats we also observed increased SAM levels and an increased ratio of SAM/SAH for trend. SAM in plasma and in CSF correlated in controls and after LPS treatment, suggesting that measurement of SAM in the plasma is suited to determine SAM availability in the CNS under physiological conditions as well as during sepsis.

Glutathione is one of the most important cellular redox systems, and oxidative stress was found to play a key role in various diseases including sepsis (29-33). During sepsis the amount of oxidized glutathione increased, the amount of reduced glutathione decreased, and,
consecutively, the ratio of oxidized/reduced glutathione increased indicating relevant oxidative stress during sepsis. In the liver about 50% of the cysteine in glutathione is derived from homocysteine via the transsulfuration pathway (34). The positive correlation between Hcys and glutathione during sepsis, but not in controls, suggests that Hcys is required for production of glutathione during sepsis (Fig1).

Transsulfuration is enhanced by high SAM levels, as SAM physiologically activates cystathionine beta-synthase (CBS) and lowers MTHFR activity (10, 22). Accordingly, we found an increase in Hcys and cysteine levels for trend. Thus, the observed data indicate that the higher levels of SAM lead to a higher production of Hcys and to a higher transsulfuration rate of Hcys to allow the synthesis of glutathione for defence of oxidative stress induced by sepsis. In addition to that, oxidative stress leads to increased flux of Hcys through the transsulfuration pathway by up-regulation of CBS activity (35) and decreased activities of methionin synthase and betaine-homocysteine S-methyltransferase (19-21). Increased transulfuration rates during experimentally induced sepsis were also shown by injections of radiolabeled cysteine and methionine in rats (36, 37).

Methionine metabolism is markedly altered during LPS-induced sepsis in rodents. The validity of our data is limited as the LPS-model of sepsis does not accurately reflect human sepsis (38). Further, blood and CSF samples were taken at one point of time only. However, the observed data encourage future studies that measure metabolites of methionine metabolism in human patients over time to explore the association of sepsis with methionine metabolism in more detail. As methionine metabolism can easily be manipulated by dietary strategies like supplementation of vitamins or SAM, such studies could lead to strategies of additive treatment of sepsis.
References


8. Greenberg SS, Xie J, Zatarain JM, Kapusta DR, Miller MJ. Hydroxocobalamin (vitamin B12a) prevents and reverses endotoxin-induced hypotension and mortality in


16. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total


Table 1. Comparison of LPS-treated and control animals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>control rats (n = 18)</th>
<th>LPS-treated rats (n = 21)</th>
<th>t-Test (two-sided)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM (nmol/L)</td>
<td>178.6 ± 33.8</td>
<td>367.2 ± 152.0</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>SAH (nmol/L)</td>
<td>39.32 ± 25.6</td>
<td>41.20 ± 28.0</td>
<td>p = 0.832</td>
</tr>
<tr>
<td>SAM/SAH ratio</td>
<td>6.55 ± 3.74</td>
<td>11.90 ± 6.39</td>
<td>p = 0.004</td>
</tr>
<tr>
<td>CSF: SAM (nmol/L)</td>
<td>324.09 ± 159.67</td>
<td>420.64 ± 141.37</td>
<td>p = 0.067</td>
</tr>
<tr>
<td>CSF: SAH (nmol/L)</td>
<td>25.14 ± 25.65</td>
<td>22.05 ± 21.62</td>
<td>p = 0.702</td>
</tr>
<tr>
<td>CSF: SAM/SAH</td>
<td>20.17 ± 11.20</td>
<td>29.87 ± 17.35</td>
<td>p = 0.068</td>
</tr>
<tr>
<td>Hcys (μmol/L)</td>
<td>2.78 ± 0.81</td>
<td>4.44 ± 2.62</td>
<td>p = 0.063</td>
</tr>
<tr>
<td>cysteine (μmol/L)</td>
<td>178 ± 15</td>
<td>218 ± 52</td>
<td>p = 0.093</td>
</tr>
<tr>
<td>GSH (nmol/mg protein)</td>
<td>216.57 ± 70.0</td>
<td>102.55 ± 38.46</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>GSSG (nmol/mg protein)</td>
<td>59.67 ± 58.36</td>
<td>117.79 ± 77.46</td>
<td>p = 0.022</td>
</tr>
<tr>
<td>GSSG/GSH</td>
<td>0.24 ± 0.17</td>
<td>0.48 ± 0.09</td>
<td>p = 0.001</td>
</tr>
</tbody>
</table>