Patients with septic shock have high plasma glutathione concentrations, whereas intracellular concentrations in erythrocytes and muscle are low. In the present study, we investigated the temporal pattern of glutathione status and glutathione kinetics in healthy volunteers during the initial phase of sepsis using a human endotoxin model. The present study was a descriptive pilot study in healthy male volunteers \((n = 8)\) before and after an endotoxin challenge. The glutathione status was determined in plasma and whole blood at baseline and hourly for 4 h after intravenous endotoxin injection and in skeletal muscle at baseline and at 2 and 4 h after endotoxin injection.

In plasma, the concentration of total glutathione decreased 24\% \((P < 0.05)\) at 3 h after endotoxin injection and 32\% \((P < 0.001)\) at 4 h. In whole blood and skeletal muscle, the concentrations of both GSH and total glutathione as well as the redox status remained unaltered during the initial 4 h after the endotoxin challenge. The FSR (fractional synthesis rate) of glutathione in whole blood was \(38 \pm 20\%\)/day before and \(59 \pm 22\%\)/day 4 h after the endotoxin challenge \((P = 0.088)\) and in skeletal muscle this was \(41 \pm 25\) and \(46 \pm 18\%\)/day \((P = 0.68)\) respectively. During the initial phase of sepsis, as represented by an intravenous endotoxin challenge to healthy volunteers, plasma concentrations of total glutathione decreased, whereas glutathione status and synthesis rate in skeletal muscle and whole blood remained unaltered. However, due to the variation in the synthesis measurements, larger studies are needed to confirm these findings.
to reference values within a week, whereas in whole blood the glutathione concentration remains at a low level [4,5]. On the other hand, plasma glutathione in critically ill patients is increased compared with healthy controls within 24 h and remains at a high concentration. Nevertheless, the temporal pattern of changes occurring in glutathione status during the initial phase of sepsis is not well characterized. In a rat model of sepsis, whole-blood glutathione concentration decreases 50%, whereas muscle glutathione concentration increases 40% 48 h after a live *Escherichia coli* challenge compared with pair-fed controls. This is accompanied by an unaltered synthesis rate in whole-blood glutathione, whereas the *de novo* synthesis rate of glutathione in muscle increases 70% [7].

To elucidate the glutathione status in humans during the early septic phase, a model where healthy volunteers are exposed to endotoxin was used. Endotoxin is a major component of the outer membrane of Gram-negative bacteria that initiates the immune response clinically manifested as the SIRS (systemic inflammatory response syndrome) [8,9]. The haemodynamic response following endotoxin administration mimics changes observed in early sepsis, although less pronounced. Nevertheless, increases in heart rate and cardiac output, and decreases in systemic vascular resistance and mean arterial pressure, are observed. The cytokine response shows a typical pattern, with an early rise in the pro-inflammatory mediators TNF-α (tumour necrosis factor-α), followed by IL-1 (interleukin)-1, IL-6 and IL-8, similar to that observed during early sepsis [8,9].

The primary aim of the present study was to characterize the glutathione status in skeletal muscle, whole blood and plasma during the initial 4 h after an endotoxin challenge in healthy volunteers. In addition, the synthesis rate of glutathione in skeletal muscle and whole blood was determined to elucidate whether a change in *de novo* glutathione synthesis was initiated.

## MATERIALS AND METHODS

### Subjects and study protocol

Healthy male volunteers (*n* = 8; age, 26 ± 3 years; weight, 74 ± 9 kg; height, 182 ± 5 cm; and BMI (body mass index), 22 ± 2 kg/m²; values are means ± S.D.) participated in the present study. All subjects were metabolically and otherwise healthy, as determined by medical history, physical examination, analyses of cell blood count and biochemical profile. The study started in the morning after an overnight fast at 08.00 hours, and lasted for 8 h. At 4 h, all subjects received an intravenous injection of U.S. Standard Reference *E. coli* endotoxin of 4 ng/kg of body weight (lot EC-6; US Pharmacopeia). The concentrations of cysteine, γ-glutamylcysteine and glutathione were measured before endotoxin injection and then hourly for 4 h in both plasma and whole blood. The skeletal muscle concentration of cysteine, γ-glutamylcysteine and glutathione was measured 2 h before and just before endotoxin injection, and then at 2 and 4 h after endotoxin injection.

Glutathione synthesis rate in whole blood and muscle was determined to elucidate whether a change in *de novo* glutathione synthesis was initiated.

---

### Table 1

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>120</th>
<th>240</th>
<th>360</th>
<th>480</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood (precursor)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole blood Glutathione enrichment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma (concentration)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole blood (concentration)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle biopsies (concentration)</td>
<td></td>
<td></td>
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</tbody>
</table>

**Endotoxin**

[Figure 1 Schematic presentation of the study protocol in healthy volunteers (*n* = 8), indicating the times of plasma and blood collection and muscle biopsies, as well as the time of endotoxin injection and tracer infusion]

---

After injection of a priming dose of 1.2 mg/kg of body weight, [13C1]glycine was infused continuously for 8 h at 1.2 mg·kg⁻¹·h⁻¹ of body weight·h⁻¹. Arterial blood samples for analyses of whole-blood glutathione concentrations and enrichment were taken before the infusion was started and then at 1 h intervals throughout the study. Blood samples for determination of free glycine isotopic enrichment were taken before tracer infusion and then every 30 min throughout the study. Glutathione synthesis in whole blood and skeletal muscle was calculated from blood samples or biopsies taken at 2 and 4 h before and 2 and 4 h after the endotoxin challenge. The study protocol is depicted in Figure 1.

Throughout the study, heart rate, ECG, oxygen saturation and blood pressure were monitored continuously using a Datex-Engstrom light monitor. Temperature was measured in the outer ear at hourly intervals.

The purpose of the study and potential risks involved were fully explained to all subjects before obtaining their
written consent. The study protocol was approved by the Ethics Committee of Karolinska Institute, Stockholm, Sweden. All subjects received financial compensation for their participation in the study.

Muscle biopsies and blood sampling
Percutaneous muscle biopsies were taken from the vastus lateralis muscle. The skin and fascia were anaesthetized by local anaesthesia with lidocaine (1 % solution). The biopsies at the later occasions were taken at least 4 cm away from the former ones. Both legs were used. The muscle tissue specimen was carefully dissected to remove all visible fat and connective tissue and then divided into separate portions to allow for the determination of cysteine, γ-glutamylcysteine and glutathione concentration and glutathione synthesis (30–40 mg of wet weight). The separate portions were weighed on an automatic electronic balance within 3 min. Biopsies were then immediately frozen in liquid nitrogen and stored at −80 °C pending analysis.

An aliquot (500 µl) of blood was immediately added to 500 µl of 2 mmol phenanthroline in 14 % perchloric acid, mixed, frozen in liquid nitrogen and stored at −80 °C.

Blood samples for plasma were centrifuged at 4000 g for 10 min at 4 °C. EDTA was used as anticoagulant. The plasma obtained was then immediately frozen in liquid nitrogen and then stored at −80 °C pending analyses.

Sample handling of muscle and blood
Frozen muscle biopsy specimens were homogenized using a mini-beat beater (Biospec Products) in 6.5 % (v/v) sulfoosalicylic acid solution. Samples were subsequently centrifuged at 3000 g for 15 min at 4 °C, resulting in the precipitation of proteins. Part of the supernatant was used for analyses of glycine enrichment, and the other part was used for determination of glutathione concentration and enrichment.

Just before analysis, whole-blood samples were treated with three freeze–thaw cycles before centrifugation at 12500 g for 15 min at 4 °C. Part of the supernatant was then used for the determination of free glycine enrichment and concentration, and the other part for glutathione enrichment and concentration.

Analyses
Both GSH (reduced glutathione) and total glutathione (after reduction with dithiothreitol), cysteine and γ-glutamylcysteine concentrations were analysed in muscle, whole blood and plasma using an HPLC technique as described previously [10]. Briefly, the pH of the supernatant was adjusted with excess sodium bicarbonate powder, and part of the sample was derivatized directly for measurement of GSH, γ-glutamylcysteine and cysteine. Total glutathione, total γ-glutamylcysteine and total cysteine were measured after reduction with dithiothreitol. Briefly, 100 µl of neutralized muscle supernatant was treated with 10 µl of 50 mmol/1 dithiothreitol, mixed and allowed to stand at room temperature (20 °C) for 30 min. Samples and standards were derivatized using monobromobimane (Calbiochem) in sodium N-ethylmorpholine.

The concentration of GSSG (oxidized glutathione), which is a dimer of the GSH, was calculated as the difference between total glutathione and GSH divided by 2. The redox status of glutathione is expressed as the ratio between GSH and total glutathione.

As plasma samples were not immediately treated with phenanthroline and precipitated with acid, only total concentrations of cysteine, γ-glutamylcysteine and glutathione were analysed in plasma samples.

The glutathione synthesis rate in whole blood and skeletal muscle was calculated from the incorporation rate of [13C1]glycine into glutathione during a continuous infusion of [13C1]glycine. The isotopic enrichment of glycine in whole blood represented the precursor pool for whole-blood glutathione synthesis. In muscle, free intracellular glycine represented the precursor pool.

The same HPLC procedure was used to isolate glutathione. GSH was isolated and then collected using a fraction collector. The isolated glutathione was then hydrolysed to its constituent amino acids at 110 °C using 0.05 M HCl for 24 h in the presence of an ion-exchange resin (AG-50 resin; Bio-Rad Laboratories). The amino acids were then eluted from the ion-exchange resin with 4 M NH₄OH and dried. Thereafter, the amino acids were derivatized with MTBSTFA (N-t-butyldimethylsilyl-N-methyltrifluoroacetamide). The tBDMS (t-butyldimethylsilyl) derivative of glycine was then analysed by GC–MS (Agilent 5973N) detecting masses m/z 246/247.

Calculations
The synthesis rate of glutathione in both whole blood and skeletal muscle is based upon the gradual increment of isotopic enrichment of glutathione-bound [13C1]glycine over time. In whole blood, the incorporation was measured at three time points during 2 h, immediately before endotoxin injection and between 2 and 4 h after injection. The incorporation rate of glutathione into whole blood was then derived from a calculated regression line of the three measured time points. The precursor pool of whole blood and skeletal muscle is whole blood and free intracellular glycine respectively. The FSR (fractio nal synthesis rate) of whole blood and skeletal muscle is calculated according to the following equation:

\[
FSR_{\text{GSH}} = \frac{E_{\text{GSHf}2} - E_{\text{GSHf}1}}{E_{\text{Gly}}} \times \frac{100}{t2 - t1} \times 12
\]

using the approach of Jahoor et al. [11], where \( E_{\text{GSHf}2} - E_{\text{GSHf}1} \) is the increase in isotopic enrichment of glutathione-bound labelled glycine over the period \( t2 - t1 \) of
Concentration of thiols in plasma during the endotoxin challenge

Concentration of thiols in whole blood during the endotoxin challenge

316 U. B. Flēring and others

+−

GSSG (GSH (Total glutathione (tGC (μmol/l)) 2.4 ± 0.3 2.3 ± 0.4 2.8 ± 1.8 2.1 ± 0.4 2.7 ± 1.8

 Values are means ± S.D. Redox, GSH/total glutathione ratio; tCYS, total cysteine; tGC, total γ-glutamylcysteine.

Table 1 Concentration of thiols in plasma during the endotoxin challenge

<table>
<thead>
<tr>
<th>Thiol</th>
<th>Baseline</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
<th>240 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>tCYS (μmol/l)</td>
<td>72 ± 16</td>
<td>68 ± 10</td>
<td>67 ± 8</td>
<td>66 ± 5</td>
<td>67 ± 9</td>
</tr>
<tr>
<td>tGC (μmol/l)</td>
<td>2.3 ± 0.3</td>
<td>2.3 ± 0.5</td>
<td>2.3 ± 0.5</td>
<td>2.9 ± 0.4</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>Total glutathione (μmol/l)</td>
<td>1.9 ± 0.9</td>
<td>1.7 ± 0.7</td>
<td>1.6 ± 0.7</td>
<td>1.4 ± 0.5**</td>
<td>1.3 ± 0.6**</td>
</tr>
</tbody>
</table>

Table 2 Concentration of thiols in whole blood during the endotoxin challenge

<table>
<thead>
<tr>
<th>Thiol</th>
<th>Baseline</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
<th>240 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>tCYS (μmol/l)</td>
<td>76 ± 8</td>
<td>74 ± 10</td>
<td>74 ± 7</td>
<td>71 ± 6</td>
<td>79 ± 17</td>
</tr>
<tr>
<td>tGC (μmol/l)</td>
<td>2.4 ± 0.3</td>
<td>2.3 ± 0.4</td>
<td>2.8 ± 1.8</td>
<td>2.1 ± 0.4</td>
<td>2.7 ± 1.8</td>
</tr>
<tr>
<td>GSH (μmol/l)</td>
<td>896 ± 153</td>
<td>904 ± 144</td>
<td>879 ± 151</td>
<td>922 ± 191</td>
<td>889 ± 175</td>
</tr>
<tr>
<td>Total glutathione (μmol/l)</td>
<td>1012 ± 159</td>
<td>1012 ± 167</td>
<td>1023 ± 162</td>
<td>1036 ± 182</td>
<td>1004 ± 176</td>
</tr>
<tr>
<td>GSSG (μmol/l)</td>
<td>67.9 ± 30.5</td>
<td>53.4 ± 28.5</td>
<td>72.1 ± 35.8</td>
<td>57.2 ± 26.6</td>
<td>57.5 ± 24.2</td>
</tr>
<tr>
<td>Redox</td>
<td>0.67 ± 0.05</td>
<td>0.89 ± 0.05</td>
<td>0.86 ± 0.07</td>
<td>0.89 ± 0.06</td>
<td>0.88 ± 0.05</td>
</tr>
</tbody>
</table>

infusion, i.e. between 2 and 4 h and between 6 and 8 h in the present study. As the time interval is 2 h, the calculated value must be multiplied by 12 to yield %/day. E_Gly is the plateau isotopic enrichment of free glycine in whole blood or muscle tissue.

ASR (absolute synthesis rate) of whole-blood glutathione was calculated from the glutathione concentration (C_GSH) and the FSR (FSR_GSH), giving the following equation:

\[ \text{ASR} = \frac{\text{C}_{\text{GSH}}}{\text{FSR}_{\text{GSH}}} \]

ASR is as expressed as μmol/l · day⁻¹ for whole blood and mmol · kg⁻¹ of body weight · day⁻¹ for skeletal muscle.

Statistics

All results are means ± S.D. The temporal changes in the concentrations of thiols in whole blood and skeletal muscle were assessed by one-way ANOVA for repeated measurements followed by Scheffe's F test. (Statistica; Statsoft). A paired Student's t test was used to compare the glutathione synthesis rate before and after endotoxin injection. All data were normally distributed according to the Kolmogorov–Smirnov test (Statistica).

RESULTS

All healthy volunteers developed similar symptoms starting approx. 1 h after administration of endotoxin. The signs and symptoms consisted of shivering, headache, muscle pain and malaise and, in some volunteers, also nausea. A low-grade fever occurred after 90 min and remained during the study period. The intensity of symptoms varied to some extent between the subjects, but was less pronounced during the last hour of the study.

Concentrations of glutathione in plasma and whole blood

Samples for plasma and whole-blood glutathione analyses were taken before and then hourly for 4 h following endotoxin injection (see the study protocol in Figure 1). The plasma concentration of total glutathione decreased 24% (P < 0.05) at 3 h after endotoxin injection and 32% (P < 0.001) at 4 h, whereas the precursors total cysteine and total γ-glutamylcysteine remained unaltered (Table 1).

In whole blood, the concentration of both GSH and total glutathione, as well as the precursors total cysteine and total γ-glutamylcysteine, remained unaltered during the study period (Table 2). In addition, the redox status of glutathione in whole blood remained unaltered.

Concentrations of glutathione in skeletal muscle

Muscle concentrations of GSH and total glutathione were analysed in specimens taken at 2 and 4 h for baseline measurement and then at 2 and 4 h after endotoxin injection (see the study protocol in Figure 1). During the study period, the concentration of both GSH and total glutathione, as well as the precursors total cysteine and total γ-glutamylcysteine, remained unaltered (Table 3). In addition, the redox status of skeletal muscle glutathione remained unaltered.

Glutathione kinetics in whole blood and skeletal muscle

In whole blood, the FSR of glutathione was 38 ± 20%/day before and 59 ± 22%/day after the endotoxin challenge (P = 0.088). The individual values are given in Figure 2. The ASR was 360 ± 177 μmol/l · day⁻¹ before
Table 3  Concentration of thiols in skeletal muscle
Values are means ± S.D. Redox, GSH/total glutathione ratio; tCYS, total cysteine; tGC, total γ-glutamylcysteine.

<table>
<thead>
<tr>
<th>Thiol</th>
<th>Control</th>
<th>Post-endotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h</td>
<td>4 h</td>
</tr>
<tr>
<td>tCYS (μmol/kg of body weight)</td>
<td>33 ± 7</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>tGC (μmol/kg of body weight)</td>
<td>2.2 ± 0.8</td>
<td>2.3 ± 0.8</td>
</tr>
<tr>
<td>GSH (μmol/kg of body weight)</td>
<td>1211 ± 194</td>
<td>1194 ± 175</td>
</tr>
<tr>
<td>Total glutathione (μmol/kg of body weight)</td>
<td>1439 ± 210</td>
<td>1412 ± 189</td>
</tr>
<tr>
<td>GSSG (μmol/kg of body weight)</td>
<td>114 ± 26</td>
<td>109 ± 20</td>
</tr>
<tr>
<td>Redox</td>
<td>0.84 ± 0.03</td>
<td>0.84 ± 0.03</td>
</tr>
</tbody>
</table>

Figure 2  FSR of whole-blood glutathione in healthy volunteers (n = 8) before and after endotoxin challenge
Results from individual subjects are shown. The bold line represents the mean values.

and 522 ± 326 μmol/l·day⁻¹ after endotoxin challenge (P = 0.10).

In skeletal muscle, the FSR of glutathione was 41 ± 25 and 46 ± 18 %/day before and after the endotoxin challenge respectively (P = 0.68). The ASR was 614 ± 410 and 688 ± 289 μmol·kg⁻¹·day⁻¹ before and after the endotoxin respectively (P = 0.68).

**DISCUSSION**

In the present study, for the first time, glutathione status and kinetics have been characterized during the initial phase of sepsis in humans using an endotoxin model. The results showed a decrease in total glutathione concentration in plasma, whereas it remained unaltered in whole blood and muscle. This decrease in plasma glutathione concentration contrasts with what is observed in patients with sepsis with multiple organ failure, where the plasma concentration of total glutathione is high compared with healthy volunteers and patients with end-stage chronic obstructive pulmonary disease [4].

Concerns may be raised that the decreased concentration of plasma glutathione observed in the present study was an effect of continued fasting. However, in a previous study conducted by our research group [12], the concentration of plasma glutathione was shown to remain unaltered during 3 days of starvation. Therefore it is unlikely that prolonged fasting influenced the decrease in plasma glutathione.

The decrease in plasma glutathione may be a consequence of an increased demand for cysteine in the activated immune system, as lymphocytes depend upon plasma glutathione for their uptake of cysteine [13]. In addition, the low plasma glutathione concentration could also reflect the uptake of glutathione, or the precursors of glutathione via γ-glutamyl transpeptidase, to maintain the intracellular glutathione pool in various tissues. This phenomenon is observed during strenuous physical exercise [14]. The high concentration of total glutathione in plasma from patients with septic shock later in the course of their disease is likely to be explained by leakage of GSSG from erythrocytes and probably also from other tissues via the endothelium [15].

In whole blood as well as in skeletal muscle, the concentrations of both GSH and total glutathione remained unaltered for the initial 4 h after the intravenous endotoxin challenge. This contrasts with ICU patients with sepsis 24 h after admission to the ICU when a glutathione depletion of approx. 40 % is observed in both of these tissues [5,16]. In addition, the redox status and the concentration of GSSG remained unaltered in both skeletal muscle and whole blood after the endotoxin challenge. This also contrasts with ICU patients with sepsis where a more oxidized glutathione status is observed [4,5,16].

There are two possible explanations for the difference with ICU patients. Either the metabolic response to endotoxin is less severe compared with patients with sepsis to result in a glutathione depletion, or, more likely, it is due to a time effect, meaning that a longer time period of sepsis or inflammation is needed for glutathione to be affected. The latter explanation is supported by animal results showing similar findings after exposure to either live bacteria or endotoxin. Both studies show that
glutathione depletion in erythrocytes does not occur until 6 h after exposure [17,18]. This suggests that glutathione depletion in erythrocytes is a slow phenomenon.

The FSR and ASR of whole-blood glutathione remained unaltered immediately following the endotoxin injection. However, the FSR and ASR increased in six out of eight subjects (Figure 2). The variation of the method turned out to be rather large, meaning that with eight subjects a power of only 0.5 was obtained. To achieve a power 0.8, at least 19 subject would be needed. In contrast, pediatric patients with sepsis studied 48 h after admission to the ICU have low values of both FSR and ASR, which might be due to a more severe insult and, in addition, that these patients with sepsis were studied at a later time point [19].

For muscle tissue, the initial time pattern of glutathione changes is less well characterized. In patients with septic shock, the depletion and the oxidized state is well-established 24 h after ICU admission, but no information regarding the temporal development is at hand from animal studies. Following moderate-size surgery, the concentrations of total glutathione and GSH decrease in muscle to the same degree as in ICU patients with sepsis (40%) at 24 h post-operatively, but without any effect upon redox status [20]. In contrast, at 6 h post-operatively, no significant changes in muscle are observed [20]. In the present study, glutathione in muscle was unaltered after the initial 4 h following endotoxin injection, although an increased efflux of glutamine and a decreased uptake of glutamate across the leg are observed within the same time frame [21]. In addition, the synthesis rates of glutathione in skeletal muscle were not changed by the endotoxin challenge; however, we show in the present study for the first time that the FSR of glutathione in human skeletal muscle is approx. 40% /day, which is of the same magnitude as in whole blood.

The concentrations of cysteine and γ-glutamylcysteine were also determined, as they are direct precursors of glutathione synthesis. Both remained unchanged during the study period, indicating that the substrate supply of cysteine for glutathione synthesis was sufficient. The utilization of glutathione increases in ICU patients with sepsis, as shown by the low concentrations in both whole blood and muscle, as well as a more oxidized redox status of glutathione, observed within the first few days of sepsis [4, 5, 19]. This also indicates the possibility that the de novo glutathione synthesis capacity is insufficient in this situation.

In summary, a decrease in plasma total glutathione concentration was observed in healthy subjects exposed to intravenous endotoxin, although the concentrations of both GSH and total glutathione, as well as the redox status of glutathione, in erythrocytes and muscle were unaltered. No changes in in vivo synthesis rates of glutathione were observed; however, the variation in these measurements was rather large. Therefore a larger study population is needed to confirm this finding.

The study suggests that very early in the course of sepsis, as represented by an endotoxin model, erythrocytes and muscle tissue are still able to maintain glutathione status.

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REFERENCES


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