Liver Glutathione and Glutathione Reductase Response of Endotoxin-Treated Mice

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The decrease in the level of liver glutathione (GSH) in endotoxin-treated mice was in part due to formation of glutathione disulfide (GSSG). An electron-generating system (EGS) had no effect when incubated with soluble liver extracts from normal controls but resulted in recovery of GSH amounting to 25% in endotoxin-treated animals. Incubation in the absence of the EGS caused a decline of 16% in the GSH in extracts from normal animals compared with a 50% decrease in endotoxin-treated animals. Exclusion of nicotinamide adenine dinucleotide phosphate (NADP) from the EGS resulted in a slight decline in the GSH of the extract from the normal controls but 25% for the endotoxin-treated animals. Reduction of exogenous GSSG by the liver extracts that exogenous NADP be added to the incubation mixtures.

Many biochemical, enzymatic, and physiological changes have been shown to occur during endotoxin shock in various experimental animals, among which has been the rapid decline in the level of reduced glutathione (GSH) in the liver (3, 11). The physiological importance of GSH has not been definitively determined, but its requirement for maintenance of erythrocyte integrity is well established (16) and, more recently, oxidized glutathione (GSSG) has been shown to interfere with the synthesis of protein by animal cells (13). In normal tissues, all the glutathione is effectively maintained in the reduced form by the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent glutathione reductase (7, 12, 14). The loss of GSH in endotoxin shock previously has been reported to be associated with inhibition of synthesis of this compound (3). However, some of the loss may be attributable to the formation of GSSG and consequent inability to catalyze its reduction to GSH. The oxidized glutathione thus formed may adversely affect reparative protein synthesis required if the shocked animal is to resist lethal effects of endotoxin. The responses of GSH and GSSG and the endogenous glutathione reductase activity in the livers of endotoxin-treated and normal control mice are reported here.

MATERIALS AND METHODS

Experimental animals. The CF-1 male mice, 20 to 25 g, obtained from Carworth Farms, Portage, Mich., were acclimated to the laboratory for 1 week before use. They were housed in metal cages with wire mesh floors and given food and water ad libitum.

Preparations for injection. Salmonella enteritidis (CDC 6601-59) endotoxin was extracted by the method of Boivin and Mesrobeanu (8) from cultures grown for 24 h on brain heart infusion (Difco) solidified with 2% agar. The lyophilized endotoxin was stored in a desiccator and for use was reconstituted with nonpyrogenic distilled water. The mean lethal dose of the endotoxin was 162.5 \( \mu g \) as determined by the method of Reed and Muench (21), with groups of six randomly selected mice used for each of six concentrations. The routine dose of endotoxin in this study was 160 \( \mu g \) administered intraperitoneally in 0.2 ml. Glutathione changes resulting from injection trauma were accounted for by injecting control mice with 0.2 ml of pyrogen-free distilled water.

Preparation of liver homogenates. After 24 h, the two mice in each experimental group were sacrificed by cervical dislocation, and their livers were excised and immediately placed in an ice bath. Approximately equal portions of each liver were blotted on filter paper and weighed on a pharmaceutical balance (Torsion). The pooled liver samples were then homogenized in 9 ml of 0.25 M mannitol per g of wet tissue with a Potter-Elvehjem (Tri-R Instruments) tissue homogenizer. The resultant homogenates were centrifuged (15,000 \( \times g \)) for 30 min at 4 C in a Sorvall RC2-B centrifuge. The supernatant fluid from each homogenate was carefully collected and maintained in an ice bath until used.

Protein determination. The protein content was determined by adding 1 ml of a 1:4 dilution of the supernatant fluid to 4 ml of biuret reagent. After thorough mixing, the reaction was allowed to proceed for 30 min at room temperature, after which the absorbance of each mixture was read at 550 nm in a spectrophotometer (Coleman Jr. II). A blank with 1
ml of distilled water replacing the supernatant fluid in the assay mixture was adjusted to 100% transmittance to correct for the color of the biuret reagent. The protein content was established by relating the absorbance to a standard curve based on bovine albumin (Sigma Chemical Co.), and this value was adjusted for the dilution.

Assay of reduced GSH. The amount of GSH present in each supernatant fluid was determined spectrophotometrically by using 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB) (Nutritional Biochemicals Corp.) in a modification of the method of Beutler et al. The protein precipitate was obtained by adding 5.15 ml of a 1:4 dilution of the protein precipitant to 1 ml of the supernatant fluid and agitated on a Vortex mixer. After 5 min in an ice bath, the precipitate was removed by filtration through Whatman no. 54 filter paper, and the clear filtrate was assayed for glutathione. The DTNB solution was prepared by dissolving 40 mg of DTNB in 100 ml of 1% aqueous sodium citrate (Fisher Scientific Co.). This solution was stable for 2 weeks at room temperature. The reaction for the GSH determination was carried out in a cuvette at room temperature by adding 2 ml of the deproteinized filtrate to 8 ml of 0.56 M Na₃H₂PO₄ and 1 ml of DTNB solution. The contents of the cuvette were mixed thoroughly, and the absorbance at 410 nm was read immediately in a spectrophotometer (Coleman Jr. II). A cuvette with 2 ml of distilled water substituted for the filtrate in the assay mixture was adjusted to 100% transmittance to correct for the inherent color of the DTNB. The absorbance readings of the filtrates were related to a standard GSH curve in terms of micrograms of GSH, and this value was converted to micromoles per milligram of protein.

GSSG purity determination. Each lot of GSSG (Nutritional Biochemicals Corp.) was assayed for purity by using yeast glutathione reductase. The assay system consisted of approximately 0.05 μmol of GSSG dissolved in 0.5 ml of distilled water, 0.5 ml of KH₂PO₄-K₂HPO₄ buffer (pH 7.6) containing 0.33 μmol of NADPH, and 0.1 ml of a 1:100 dilution of yeast glutathione reductase (Sigma). The total volume (1.1 ml) was the same as used for experimental determinations. Incubation for 5 min at 30 C was sufficient to allow the total reduction of the GSSG in the assay mixture. After incubation, the protein was precipitated and the amount of GSH present in the system was determined by the routine GSH assay. A solution was then prepared in distilled water in concentrations based upon the assayed GSSG content, divided into portions, and frozen until used. The observations of GSSG reduction by the supernatant fluids were made with this solution.

Glutathione reductase activity. The assay of glutathione reductase activity was performed by incubating 1 ml of the supernatant fluids from the liver homogenates with 0.1 ml of an NADPH-generating system at 30 C for 15 min. The reaction was stopped by transferring the incubation mixture to an ice bath and immediately adding ice-cold deprenoylizing solution. A portion of each supernatant fluid held in an ice bath served as an unincubated control, and another portion was incubated at 30 C with 0.1 ml of distilled water replacing the electron-generating system (EGS).

The EGS used in the assay of glutathione reductase contained glucose-6-phosphate (G-6-P; 0.8 mg) (General Biochemicals), nicotinamide adenine dinucleotide phosphate (NADP; 0.19 mg) (Pabst), and nicotinamide (0.1 mg) (Nutritional Biochemicals) in 0.1 ml. This solution was stored at −10 C until used.

The efficiency of the liver glutathione reductase of the supernatant fluids was measured by the ability of each to reduce a specified amount of exogenous GSSG. A 1-ml portion of each supernatant fluid was incubated with the EGS for 15 min at 30 C to measure their total endogenous GSH content. Another portion, which received 0.1 ml of the EGS containing 0.023 μmol of GSSG, was also incubated at 30 C for 15 min. The efficiency of exogenous GSSG reduction was determined by comparing the increase in GSH above the endogenous level with the theoretical increase predicted on the basis of 100% reduction.

RESULTS

The mean GSH level of the liver from endotoxin-treated mice was 1.29 μmol per mg of protein or about 53% of the normal (Table 1). In addition to the decline in the GSH level observed in the livers of endotoxin-treated mice, the GSH level in the homogenate also was more susceptible to environmental stress. Incubation of the normal homogenate at 30 C for 15 min resulted in a decline of only 16% in GSH level compared with a 50% decrease in the homogenate from the endotoxin-treated mice. By contrast, when the homogenates were incubated under the same conditions with an NADPH-generating system (EGS), the GSH level of the liver homogenates from normal and endotoxin-treated mice

**Table 1. Effect of an electron-generating system on the GSH level of liver homogenates from normal and endotoxin-treated mice**

<table>
<thead>
<tr>
<th>Incubation system</th>
<th>μmol of GSH/mg of protein (×10⁻² ± Standard deviation)</th>
<th>Normal</th>
<th>Endotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (iced)</td>
<td>2.41 ± 0.16</td>
<td>1.29 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>Incubated</td>
<td>2.01 ± 0.21</td>
<td>0.65 ± 0.29</td>
<td></td>
</tr>
<tr>
<td>NADPH</td>
<td>2.41 ± 0.16</td>
<td>1.61 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>NADPH + GSSG</td>
<td>2.61 ± 0.16</td>
<td>1.82 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>NADPH*</td>
<td>2.08 ± 0.25</td>
<td>0.69 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>NADPH + GSSG*</td>
<td>1.99 ± 0.24</td>
<td>0.69 ± 0.34</td>
<td></td>
</tr>
</tbody>
</table>

* Components added after deprenoylization.
level of the normal homogenate was unchanged from the normal control values, but the homogenates from the endotoxin-treated animals gained a level of GSH amounting to approximately 25%. Addition of the EGS to the deproteinized, incubated homogenates resulted in no change from the incubated homogenates. Exogenous GSSG added with the EGS was reduced to GSH by the homogenates from both the normal and the endotoxin-treated mice. Addition of the GSSG and EGS after deproteinization resulted in a slight decrease in the GSH level in the normal homogenate, but in the homogenate from the endotoxin-treated animal the level was the same as that of the comparable test system. 

Exogenous GSSG was completely reduced to GSH by the homogenates from both the normal and endotoxin-treated mice, with recovery being slightly greater than unity after 15 min of incubation. The average reduction of GSSG to GSH was 18.0 μg for normal controls and 15.4 μg for the endotoxin-treated mice when 14.1 μg (0.023 μmol) of GSSG and EGS was added to the supernatant fluids. The reduction of exogenous GSSG was more than 90% complete within 5 min and was completed within 10 min (Fig. 1). In both cases the rate at which the exogenous GSSG was reduced was comparable.

To show that the loss of GSH during incubation was due to simple oxidation, the homogenates were first incubated for 15 min, the EGS was then added, and the mixture was reincubated for another 15 min. The GSH levels determined in these experiments were comparable to those in which the EGS was added initially and incubated for only 15 min (Table 2).

The role of the various components of the EGS was determined by incubating homogenates with different deletions. In the absence of NADPH the GSH level in the normal homogenate declined 8%, but incubation of the endotoxin homogenate without the NADP in the EGS resulted in a 30% decline in GSH, a value comparable to that obtained with the incubated control (Table 3). Exclusion of G-6-P from the EGS failed to influence the ability of the homogenates to reduce the NADP as shown by reduction of the GSSG to GSH. Exogenous GSSG added to these incubation mixtures was

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**Table 2. Effect of an electron-generating system, added at different times, on the GSH level of liver supernatant fluids from normal or endotoxin (160 μg)-treated mice**

<table>
<thead>
<tr>
<th>Incubation system</th>
<th>μmol of GSH/mg of protein (×10⁻² ± Standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Control (iced)</td>
<td>2.47 ± 0.51</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2.10 ± 0.47</td>
</tr>
<tr>
<td>EGS initial*</td>
<td>2.46 ± 0.58</td>
</tr>
<tr>
<td>EGS after*</td>
<td>2.51 ± 0.52</td>
</tr>
</tbody>
</table>

* EGS was added to the supernatant fluid and the mixture was incubated for 15 min.

* Supernatant fluid was incubated for 15 min, the EGS added, and the mixture reincubated for 15 min.

**Table 3. Effect of individual components of the electron-generating system on the conservation of the liver glutathione in supernatant fluids obtained from normal and endotoxin (160 μg)-treated mice**

<table>
<thead>
<tr>
<th>Incubation system</th>
<th>μmol of GSH/mg of protein (×10⁻² ± Standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Control (iced)</td>
<td>2.75 ± 0.21</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2.58 ± 0.22</td>
</tr>
<tr>
<td>EGS only</td>
<td>2.96 ± 0.23</td>
</tr>
<tr>
<td>EGS + GSSG</td>
<td>3.29 ± 0.20</td>
</tr>
<tr>
<td>EGS – NADP</td>
<td>2.72 ± 0.28</td>
</tr>
<tr>
<td>EGS – NADP + GSSG</td>
<td>2.72 ± 0.32</td>
</tr>
<tr>
<td>EGS – G-6-P</td>
<td>2.95 ± 0.26</td>
</tr>
<tr>
<td>EGS – G-6-P + GSSG</td>
<td>3.27 ± 0.23</td>
</tr>
<tr>
<td>EGS – NADP – G-6-P</td>
<td>2.68 ± 0.31</td>
</tr>
<tr>
<td>EGS – NADP – G-6-P+GSSG</td>
<td>2.65 ± 0.27</td>
</tr>
<tr>
<td>GSSG only</td>
<td>2.69 ± 0.22</td>
</tr>
</tbody>
</table>
affected in the same way as observed for the endogenous GSSG.

DISCUSSION

*S. enteritidis* endotoxin has been shown previously to cause a 24% decrease in the liver nonprotein sulphydryl (NPSH) level within 2 h, with a further decrease to 40% after 18 h (11). A similar effect after treatment with *Serratia marcescens* endotoxin was observed by Beck and Linkenheimer (3). Mice stressed by hind-leg ligation, exposure to cold, or scalding displayed markedly decreased NPSH levels. Injections of serotonin (9), epinephrine (22), and chlorpromazine (2, 15) elicited a similar response. Since GSH comprises 90% of the liver NPSH (12), these changes in tissue NPSH are changes in the GSH level. Szymanski and Jeffries (25) attempted to replace the GSH lost during endotoxin shock by administering several doses of reduced glutathione. The treatment of endotoxin shock with GSH was unsuccessful, but pretreatment with GSH (30 mg) 2 to 4 h before *S. enteritidis* endotoxin resulted in a significantly improved survival of the test mice.

Because of the susceptibility of GSH to oxidation under many conditions, it was considered that this might account for the decrease in the GSH in endotoxin-poisoned mice. Therefore, an EGS was added to the various supernatant fluids to mediate the reduction of endogenous GSSG to GSH. This treatment did not appreciably increase the GSH level of normal animals. In contrast, incubation of the endotoxin supernatant fluid with the EGS increased the total GSH level by 25%. This was particularly noteworthy since GSH is synthesized (12, 24) and exists in mammalian tissues primarily in the reduced form (12, 14). However, reduction of the endogenous GSSG in the endotoxin supernatant fluid restored the total GSH level to only two-thirds that of the normal control. Thus, endotoxin-treated mice differed from the normal mice in both their total GSH content and the distribution of the GSH between its oxidized and reduced forms.

GSH can be oxidized either by molecular oxygen or enzymatically by glutathione peroxidase (16). The disappearance of GSH and the formation of GSSG during aerobic incubation are almost quantitatively related (18), and the loss of GSH increases with incubation time (17). In the present study, the loss of endogenous GSH during aerobic incubation in the absence of the EGS was greatest in endotoxin-treated mice. Since this GSH was recovered by incubation with the EGS, it can be assumed that the endotoxin supernatant fluid possessed a fully functional glutathione reductase.

Subsequent experiments were designed to demonstrate the ability of the endotoxin supernatant fluid to reduce a specified quantity of exogenous GSSG at a rate comparable to that of the normal control. The GSH level decreased to below the endogenous value during the first minute of exposure to exogenous GSSG. This effect was most pronounced in the endotoxin supernatant fluid and possibly reflects a transient inhibition of glutathione reductase activity. Pinto (17) has reported that the rate of aerobic GSSG reduction in pigeon liver homogenates decreased as the GSSG concentration was increased. These experiments further indicate that despite the lowered hepatic GSH content, endotoxin-treated mice possess a functional glutathione reductase. However, endotoxin shock may affect the cofactors required for glutathione reductase activity.

Omission of G-6-P from the EGS had no effect on the final level of GSH in the supernatant fluids. This result was unexpected since it was anticipated that the limited availability of carbohydrates in the liver of endotoxin-treated mice would decrease the quantity of electrons available for glutathione reductase activity. This finding, however, indicates that a sufficient alternate source of electron donors exists in the liver to compensate for the anticipated loss of G-6-P. Based on studies of erythrocyte glutathione reductase, Beutler and Yeh (6) have suggested that because of its lower oxidation-reduction potential, the G-6-P—6-phosphogluconate system is thermodynamically better suited to GSSG reduction than the lactate-pyruvate system. It may be that G-6-P is the preferred electron donor, but when glycolytic metabolism predominates, as results from endotoxin-induced mobilization of liver glycogen, other electron-generating systems such as those mediated by lactic dehydrogenase, phosphoglyceraldehyde dehydrogenase, etc., may become the primary source of electrons for NADPH formation. Rieber et al. (23) have reported that NADH generated by the action of lactate dehydrogenase can mediate endogenous GSSG reduction in homolysates from erythrocytes deficient in G-6-P dehydrogenase activity. Other alternate electron donors may also function to some extent in nonglycolytic situations, especially if the supply of G-6-P is limited. Pinto (17) has reported that the addition of G-6-P to pigeon liver homogenate increased the GSSG reduction 1.5 times above the endoge-
nous rate. However, citrate and fumarate proved to be twice as effective as electron donors for GSSG reduction.

In contrast with G-6-P, exclusion of NADP from the incubation mixture resulted in a decrease in the GSH level in both normal and endotoxin supernatant fluids. However, only in the endotoxin supernatant fluid did this decrease represent a significant portion of the total liver GSH. NADPH has been shown essential for liver glutathione reductase activity (20), and in its presence the accumulation of GSSG is antagonized. The rate of GSSG reduction by glutathione reductase has been reported to be independent of the GSSG concentration, although there may be an inhibitory effect at high GSSG levels (17). Since endotoxin-treated mice have been shown to possess a functional glutathione reductase and a sufficient supply of electron donors, the lowered GSH levels may be presumed to be the result of an inadequate supply of NADP for the reduction of GSSG. The report of Berry and Smythe (4) that the total pyridine nucleotide level in the liver 17 h after an LD₉₀ of endotoxin was two-thirds that of the untreated controls lends support to this interpretation. In addition, in the absence of added NADP neither the normal nor the endotoxin supernatant fluid was able to reduce exogenous GSSG. The normal supernatant fluids incubated without added NADP and those maintained in ice had comparable GSH levels. Therefore, the inability of the normal supernatant fluid to reduce the exogenous GSSG in the absence of added NADP may indicate that, in vivo, little NADP is available to react with glutathione reductase. In pigeon liver homogenates, the rate of aerobic GSSG reduction is comparable to the anaerobic rate only when exogenous NADP is added (17). This also would indicate that the endogenous NADP level is rate limiting for GSSG reduction. In contrast, the GSH level in the endotoxin supernatant fluid incubated without added NADP was significantly lower than its iced control. Therefore, the absence of GSSG reduction in the endotoxin supernatant fluid may very well reflect an aggravation of the assumed NADP deficiency.

Nicotinamide was added to the EGS to protect the NADP by preventing nicotinamide-nucleotide cleavage described by Zatman et al. (26). Nicotinamide has been reported to inhibit the formation of GSSG by maintaining the reduced state of the nicotinamide nucleotides (19).

Glutathione does not appear to be a primary target for endotoxin action, although the endotoxin-associated decrease in liver pyridine nucleotides profoundly influences the liver GSH level. The inadequate supply of NADP for glutathione reductase activity and the inhibition of tripeptide synthesis appear to account for the loss of liver glutathione after the administration of endotoxin. Since glutathione maintains enzymes in their reduced (active) form, reduces hydroperoxides to hydroxylated lipids or hydroxylated fatty acids (10, 19), etc., a decrease in its level can result in a variety of metabolic derangements. Because endotoxin is also known to produce profound physiological and metabolic alterations in the host, the effect of endotoxin on liver glutathione, even if only a secondary manifestation, merits further study.

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LITERATURE CITED