Dietary sulphur amino acid adequacy influences glutathione synthesis and glutathione-dependent enzymes during the inflammatory response to endotoxin and tumour necrosis factor-α in rats

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1. Glutathione concentrations in liver and lung fall when food intake or sulphur amino acid intake is inadequate. However, concentrations may be restored during inflammation, despite anorexia, provided that prior sulphur amino acid intake is adequate.

2. We studied the mechanisms of these changes by measuring the effect of sulphur amino acid and protein intake on hepatic glutathione synthesis and γ-glutamylcysteine synthetase activity, hepatic and lung glutathione concentrations, glutathione reductase and glutathione peroxidase activities in young rats given an inflammatory challenge by intraperitoneal injection of tumour necrosis factor-α or endotoxin (lipopolysaccharide).

3. Diets containing 200 g of casein and 8 g of L-cysteine/kg (normal-protein diet), or 80 g of casein and 8 g of L-cysteine, or isonitrogenous amounts of L-methionine or L-alanine (low-protein diets) were fed ad libitum to young Wistar rats for 8 days. Dietary groups were subdivided into three: one subgroup continued feeding ad libitum, a second was given tumour necrosis factor or lipopolysaccharide and killed 24 h thereafter, while the third was pair-fed to the intakes of the second subgroup for 24 h before being killed.

4. Glutathione concentrations in liver and lung were reduced in rats fed the low-protein diet containing alanine, and in all dietary groups when food intake was restricted. The inflammatory challenges restored hepatic glutathione concentrations in all groups but the diet supplemented with alanine, which had an inadequate sulphur amino acid content. In lung, restoration occurred only in animals fed the normal-protein diet.

5. The activity of γ-glutamylcysteine synthetase, which is rate limiting for glutathione synthesis, was unaffected by dietary or sulphur amino acid intake or by the inflammatory response. Substrate supply may therefore be a major determinant in glutathione synthesis in vivo.

6. Total hepatic glutathione synthesis was affected by food intake, the type and amount of sulphur amino acids in the diet and by inflammation. Total synthesis was 207, 137, 421 and 90 μmol/day for animals fed ad libitum the normal-protein diet, or low-protein diets supplemented with cysteine, methionine or alanine respectively, ad libitum. Pair-feeding resulted in values of 76, 31, 71, and 0 μmol/day respectively. After lipopolysaccharide injection, rates increased to 200, 117, 151 and 56 μmol/day respectively.

8. Reductase and peroxidase activities increased in liver and lung, when low-protein diets which contained supplemental methionine or alanine were consumed ad libitum. A reduction in food intake resulted in enzyme activity changes, which suggested that recycling of glutathione increased in lung and decreased in liver. Injection of tumour necrosis factor reversed this effect.

9. The restoration of glutathione concentrations in liver after an inflammatory challenge is closely associated with an enhanced rate of synthesis and increased recycling. The former is impaired when inadequate sulphur amino acid is consumed before the challenge. In lung, increased recycling of glutathione may help maintain concentrations when food intake is restricted, but not during inflammation.

INTRODUCTION

Glutathione is the main sulphydryl compound in mammalian cells. It is present in millimolar quantities and is predominantly found in the reduced form (GSH) as a tripeptide of cysteine, glycine and glutamic acid [1]. The oxidized form of glutathione (GSSG) consists of two tripeptide molecules linked by a disulphide bridge. Glutathione is synthesized...
from its constituent amino acids, predominantly by the liver and kidney, by a two-step metabolic pathway. In the first step glutamic acid and cysteine are converted into γ-glutamylcysteine. The second step is rate limiting and converts γ-glutamylcysteine into GSH. Glutathione has a number of important functions in metabolism. It is a major component of cellular antioxidant defences and participates in the detoxification of xenobiotics [2].

Glutathione acts as a major source of cysteine for lymphocytes [3–5]. The normal functioning of T-lymphocytes is dependent upon cellular supplies of cysteine. The cells acquire the amino acid largely by uptake of GSH by macrophages and transferal of the cysteine moiety to lymphocytes. Impaired immune responses are associated with a reduction in the glutathione concentration of immune tissues [4, 6].

The relative demands for the sulphur amino acids may increase during the inflammatory response. Marked increases in the excretion of nitrogen- and sulphur-containing compounds in urine occurs in trauma and infection as a consequence of enhanced efflux of amino acids from peripheral tissue and increased hepatic amino acid metabolism. A fall in the ratio of sulphur to nitrogen in urine, during the inflammatory response, suggests that a preferential retention of sulphur amino acids may occur [7–9]. Hepatic glutathione concentrations are lowered by a reduction in dietary protein intake and elevated by retention of sulphur amino acids may occur [7–9]. Hepatic glutathione concentrations are lowered by a reduction in dietary protein intake and elevated by retention of sulphur amino acids may occur [7–9].

In normal rat liver similar amounts of cysteine are present in protein and glutathione, 34.4 and 20.0 μmol/g of tissue wet weight respectively. This estimate is derived from the observation that rat liver protein contains 133.2 μmol/g [13] and GSH contains 2.98 mmol/g, and assumes a concentration of 258 μg of protein/g of tissue and a GSH concentration of 6.71 mg/g of tissue, as observed in an earlier study employing similar diets to those reported here [11]. Thus relative and absolute changes in the rate of synthesis of either of these tissue constituents will influence the requirements for sulphur amino acids.

In rats fed diets of low protein content, an impaired metabolic response to the proinflammatory cytokine, tumour necrosis factor-α (TNF-α) has been found [11, 14–16]. Features of the impaired response were an inability to increase hepatic protein, GSH and serum α2 macroglobulin to the same extent as in rats fed a diet of adequate protein content. Addition of cysteine and methionine to the low-protein diet restored these responses towards normal, indicating that the impairment was due to dietary sulphur amino acid insufficiency. The increase in total liver protein synthesis in response to inflammation was however influenced to a much lesser extent by dietary sulphur amino acid deficiency than the ability to increase GSH concentration [11]. The question therefore arises as to whether sulphur amino acid deficiency impairs the synthesis of GSH to a much greater extent than that of protein [9].

While various estimates of tissue glutathione synthesis have been made in unstressed animals, the measurement of glutathione synthetic rates during inflammation has received little attention. Where measured, conflicting results have been obtained, suggesting both increases and decreases in the rates of glutathione synthesis in response to an inflammatory stimulus [12, 17].

This paper examines the influence of dietary sulphur amino acid intake on liver and lung glutathione concentration, the activities of enzymes involved in glutathione metabolism and the rate of hepatic glutathione synthesis in young rats, in the presence and absence of inflammatory stimuli. The stimuli were in the form of TNF for the enzyme studies and endotoxin [lipopolysaccharide (LPS)] for a kinetic study in which rates of glutathione synthesis were measured. The enzyme study examines the effect of consuming a low-protein diet, supplemented with isonitrogenous amounts of either cysteine, methionine or alanine, on the activities of enzymes associated with glutathione metabolism, and the influence of TNF on these enzymes in liver and lung. The kinetic study examines the rate at which glutathione is synthesized by liver under the same dietary conditions and in response to LPS injection. Since anorexia is a common feature of the response to both TNF and LPS, the studies also included pair-fed groups to reveal the influence of a reduction in food intake on the metabolic response.

**MATERIALS AND METHODS**

Animal procedures were conducted in accordance with legislation laid down by the Home Office of the British Government.

**Enzyme study**

Young male Wistar rats from the Southampton University Medical School colony (weight 99 ± 7 g) were fed for 8 days on diets in which the sole source of protein was edible and washed casein (Besnier Bridel Alementaire, 35230, Bourgbarre, France). The diets were a normal-protein diet (200 g of casein/kg) supplemented with L-alanine and L-cysteine or one of a group of low-protein diets (80 g of casein/kg) supplemented with isonitrogenous amounts (2.8 mg of N/kg) of L-alanine, L-cysteine or L-methionine (Table 1). Animals from each dietary group received either 50 μg of recombinant human TNF (BASF/Knoll AG, Ludwigshafen, Germany) in sterile saline per kilogram of body weight (day 8, TNF group) or sterile saline alone (day 9, pair-fed group) by the intraperitoneal route while continuing their diets. The TNF injections resulted in a loss of appetite.
Table 1. Composition of diets. *Vitamin and mineral mix (AIN-76, American Institute of Nutrition 1977).

<table>
<thead>
<tr>
<th></th>
<th>Normal-protein diet (g/kg)</th>
<th>Low-protein diets (g/kg)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cysteine</td>
<td>Methionine</td>
<td>Alanine</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>8</td>
<td>8</td>
<td>—</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>—</td>
<td>—</td>
<td>10</td>
</tr>
<tr>
<td>Vitamin mix*</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mineral mix*</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Sucrose</td>
<td>295</td>
<td>335</td>
<td>354</td>
</tr>
<tr>
<td>Maize starch</td>
<td>295</td>
<td>335</td>
<td>354</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Maize oil</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

during the 24 h after injection, therefore during that period saline-injected controls were pair-fed an amount of food identical with that consumed by an animal of similar weight receiving TNF in the same dietary group.

Animals were killed 24 h after injection, or on day 10 for control animals (who consumed diet at an ad libitum rate for the period of the study), by stunning and decapitation. Liver and lungs were rapidly dissected and frozen in liquid nitrogen. Before freezing, small samples of liver and lung were taken for measurement of GSH concentration by colorimetric reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) [18]. Tissue samples for measurement of enzymes of glutathione metabolism were prepared by homogenization and sonication.

Liver and lung glutathione reductase (EC 1.6.4.2) was measured in the presence of GSSG by following the oxidation of NADPH spectrophotometrically at 340 nm [19]. Liver and lung glutathione peroxidase (EC 1.11.1.9) was measured in the presence of GSH by following the disappearance of NADPH at 366 nm [20]. Liver γ-glutamylcysteine synthetase (EC 6.3.2.2) was measured by the coupled enzyme assay of Seelig and Meister [21], which utilized pyruvate kinase and lactate dehydrogenase. Tissue protein concentrations were measured by the biechinonic acid method [22].

Kinetic study

Young male Wistar rats from the Southampton University Medical School colony (weight 97 ± 5 g) were fed as detailed in the enzyme study. Animals from each dietary group received either 0.1 μg of LPS in sterile saline per kg of body weight (day 8, LPS group) or sterile saline alone (day 9, pair-fed group) by the intraperitoneal route while continuing their diets. The saline-injected controls were pair-fed on day 9 as detailed in the enzyme study. Animals were killed 24 h after injection, or on day 10 for controls fed ad libitum, by stunning and decapitation. Before death, liver glutathione synthesis rates were determined using d,L-buthionine-S-sulphoximine (BSO) [23]. The method assumes that glutathione synthesis is completely inhibited by BSO while utilization proceeds at the rate at the time of injection with BSO. The rate of glutathione disappearance therefore reflects the rate of glutathione synthesis, on the assumption that synthesis matches utilization over short periods of time, such as the 3 h period over which measurements are made in the BSO technique. Tissue glutathione concentration was measured by the method of Beutler et al. [18].

Statistical analysis

Results are given as means ± pooled SEM. The results were compared using one-way analysis of variance or two-way analysis of variance with treatment group and diet as independent variables with a significance level of P<0.05. Where significant effects were found, differences between groups were examined by an unpaired Student’s t-test with a significance level of P<0.05.

RESULTS

Growth and tissue composition

Results from both the enzyme study (Tables 2, 4, 6 and 8) and kinetic study (Tables 3, 5 and 7) illustrate the effects of feeding a low-protein diet with or without sulphur amino acid supplementation, on growth, tissue weight, glutathione concentration, glutathione-dependent enzyme activities and glutathione synthesis rates in young rats. Growth was severely impaired by the low-protein diet supplemented with alanine. Addition of sulphur amino acids restored growth towards rates seen in animals fed the normal-protein diet. Cysteine was more effective than methionine in this respect (Tables 2 and 3). Administration of inflammatory agents reduced food intake. However, food intakes were reduced to a greater extent in the TNF-treated animals compared with the animals given LPS. Consequently, both the TNF-treated and pair-fed groups lost more weight in the 24 h after treatment in the enzyme study compared with the LPS-treated animals in the kinetic study. Diets containing 6 g/kg of cysteine and 6 g/kg of methionine have been recommended to meet the sulphur amino acid requirements of young rats [24]. However, a dietary content of 1.7 g of methionine/kg and 2.4 g of cystine/kg has also been shown to be adequate to meet requirements for growth [25]. The dietary sulphur amino acid content in the latter study was 32.4 mmol/kg. Casein contains 35.5 and 185 mmol/kg of cysteine and methionine respectively (values provided by the manufacturer). Thus in the present study the normal diet containing 200 g of casein and
Table 2. Growth of rats fed low-protein diets supplemented with cysteine, methionine and alanine, and the effect of TNF on body weights and food intake. Values are means, n = 5 per group. Within a row, values with different letter superscripts differ significantly (P < 0.05). *TNF or pair fed is different from ad libitum value (P < 0.05).

<table>
<thead>
<tr>
<th>Normal-protein diet</th>
<th>Cysteine</th>
<th>Methionine</th>
<th>Alanine</th>
<th>Pooled SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth before injection (g/day)</td>
<td>7.6a</td>
<td>4.3a</td>
<td>2.9a</td>
<td>0.4a</td>
</tr>
<tr>
<td>Change in body weight after treatment (g/24 h)</td>
<td>5a</td>
<td>4a</td>
<td>5a</td>
<td>2b</td>
</tr>
<tr>
<td>Ad libitum</td>
<td>4a</td>
<td>5a</td>
<td>2b</td>
<td>0.9</td>
</tr>
<tr>
<td>Pair fed</td>
<td>-11b**</td>
<td>-14b**</td>
<td>-3b</td>
<td>1.8</td>
</tr>
<tr>
<td>TNF</td>
<td>-15b**</td>
<td>-13b**</td>
<td>-3b**</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Food intake 24 h post-treatment (g/24 h)

<table>
<thead>
<tr>
<th>Normal-protein diet</th>
<th>Cysteine</th>
<th>Methionine</th>
<th>Alanine</th>
<th>Pooled SEM</th>
</tr>
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<tr>
<td>Ad libitum</td>
<td>27a</td>
<td>22a</td>
<td>13a</td>
<td>0.4</td>
</tr>
<tr>
<td>Pair fed LPS</td>
<td>5a**</td>
<td>2a**</td>
<td>9a</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Table 3. Growth of rats fed low-protein diets supplemented with cysteine, methionine and alanine, and the effect of endotoxin (LPS) on body weights and food intake. Values are means, n = 4 per group. Within a row, values with different letter superscripts differ significantly (P < 0.05). *LPS or pair fed is different from ad libitum value (P < 0.05).

<table>
<thead>
<tr>
<th>Normal-protein diet</th>
<th>Cysteine</th>
<th>Methionine</th>
<th>Alanine</th>
<th>Pooled SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth before injection (g/day)</td>
<td>7.4a</td>
<td>4.6b</td>
<td>3.6b</td>
<td>1b</td>
</tr>
<tr>
<td>Change in body weight after treatment (g/24 h)</td>
<td>5a</td>
<td>6a</td>
<td>7a</td>
<td>7a</td>
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<tr>
<td>Ad libitum</td>
<td>4a</td>
<td>7a</td>
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<td>1.5</td>
</tr>
<tr>
<td>Pair fed LPS</td>
<td>-4a**</td>
<td>-2a**</td>
<td>-2a**</td>
<td>2.0</td>
</tr>
<tr>
<td>Food intake post-LPS injection (g/24 h)</td>
<td>29a</td>
<td>23a</td>
<td>22a</td>
<td>1.5</td>
</tr>
<tr>
<td>Ad libitum</td>
<td>13a</td>
<td>12a</td>
<td>13a</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Table 4. Influence of cysteine and methionine supplementation on the action of TNF on liver weight of rats fed low-protein diets. Values are means, n = 5 per group. Within a row, values with different letter superscripts differ significantly (P < 0.05). *TNF or pair fed is different from ad libitum value (P < 0.05). †TNF is different from pair fed (P < 0.05).

<table>
<thead>
<tr>
<th>Normal-protein diet</th>
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<th>Alanine</th>
<th>Pooled SEM</th>
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</thead>
<tbody>
<tr>
<td>Liver (g)</td>
<td>9.6a</td>
<td>8.4b</td>
<td>7.1b</td>
<td>4.6b</td>
</tr>
<tr>
<td>Ad libitum</td>
<td>6.1b</td>
<td>5.5b**</td>
<td>5.1**</td>
<td>3.4**</td>
</tr>
<tr>
<td>Pair fed TNF</td>
<td>50.9†</td>
<td>49.0**†</td>
<td>47.6**†</td>
<td>50.4**†</td>
</tr>
</tbody>
</table>

8 g of cysteine/kg contained 110 mmol of sulphur amino acids/kg. The low-protein diets containing 80 g of casein/kg, with additions of cysteine, methionine and alanine contained 84, 85 and 18 mmol respectively of sulphur amino acids/kg. Thus dietary sulphur amino acid adequacy was not achieved in the groups consuming the low-protein diet supplemented with alanine, or when the diets were fed in restricted amounts for the 24 h before sacrifice.

Pair-fed animals from both studies had smaller relative liver weights compared with the corresponding controls fed ad libitum (Tables 4 and 5). The reduction in liver size was largely overcome by the effects of TNF or LPS, such that these groups had significantly larger livers compared with their pair-fed counterparts in seven out of the eight groups in the two studies. The fall in liver size due to pair-feeding, and the prevention of the fall by the inflammatory stimulus, was mirrored in the liver glutathione concentrations. However, a clear distinction was evident, in each study, between the magnitude of the responses in the four dietary groups.
Table 5. Influence of cysteine and methionine supplementation on the action of endotoxin (LPS) on liver weight of rats fed low-protein diets. Values are means, n = 4 per group. Within a row, values with different letter superscripts differ significantly (P < 0.05). *LPS or pair fed is different from ad libitum value (P < 0.05). †LPS is different from pair fed (P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Normal-protein diet</th>
<th>Low-protein diets</th>
<th>Cysteine</th>
<th>Methionine</th>
<th>Alanine</th>
<th>Pooled SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>8.1*</td>
<td>7.0*</td>
<td>7.5°</td>
<td>4.5°</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Pair fed</td>
<td>6.5**</td>
<td>5.2**</td>
<td>5.9°*</td>
<td>3.6°*</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>7.3°</td>
<td>6.9†</td>
<td>6.4†</td>
<td>4.7†</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Liver (g/kg of body wt.)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>49.2b</td>
<td>54.4*</td>
<td>55.3*</td>
<td>41.4*</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Pair fed</td>
<td>49.1mc</td>
<td>38.7**</td>
<td>48.1**</td>
<td>35.3*</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>48.9m†</td>
<td>52.7b</td>
<td>52.6†</td>
<td>44.6†</td>
<td>1.7</td>
<td></td>
</tr>
</tbody>
</table>

Animals consuming a normal-protein diet, or low-protein diets supplemented with sulphur amino acids ad libitum, had significantly greater liver and lung glutathione concentrations than the group fed the low-protein diet supplemented with alanine. Methionine supplementation resulted in the highest concentration of GSH in both tissues when the diets were fed ad libitum. Concentrations in both tissues fell in all groups when food intake was restricted, such that the difference between concentrations in the animals fed the diet supplemented with alanine and the other dietary groups was no longer evident. The difference re-emerged when an inflammatory stimulus was applied to the animals (Tables 6 and 7).

In liver and lung there were similarities and differences in the effect of TNF administration on GSH concentration. In animals fed the normal-protein diet, GSH concentrations returned to values found in animals fed ad libitum, in both tissues. In liver, values in groups given TNF were significantly greater than in their pair-fed counterparts. However the magnitude of the difference was less in animals fed low-protein diets supplemented with alanine than when supplemented with sulphur amino acids. In the groups receiving the low-protein diets supplemented with cysteine and methionine, values remained lower than those observed when the diets were fed ad libitum. In lung there was no difference in values for the groups given TNF and those which were pair fed, when the diet had a low protein content.

Activities of glutathione-dependent enzymes

The activities of enzymes associated with glutathione metabolism are shown in Tables 6 and 8.

Table 6. Influence of cysteine and methionine supplementation on the action of TNF on liver glutathione, glutathione peroxidase, glutathione reductase and γ-glutamylcysteine synthetase of rats fed low-protein diets. Values are means, n = 5 per group. Within a row, values with different letter superscripts differ significantly (P < 0.05). *TNF or pair fed is different from the ad libitum value (P < 0.05). †TNF is different from pair fed (P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Normal-protein diet</th>
<th>Low-protein diets</th>
<th>Cysteine</th>
<th>Methionine</th>
<th>Alanine</th>
<th>Pooled SEM</th>
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<tbody>
<tr>
<td>Glutathione (μmol/g)</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>7.9b</td>
<td>9.1*</td>
<td>10.1*</td>
<td>2.7*</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Pair fed</td>
<td>2.1**</td>
<td>2.1**</td>
<td>2.1**</td>
<td>2.2*</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>6.1†</td>
<td>6.1†</td>
<td>5.8**†</td>
<td>2.9†</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase (units/g of protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ad libitum</td>
<td>41b</td>
<td>38b</td>
<td>56b</td>
<td>61b</td>
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<tr>
<td>Pair fed</td>
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<td>46b</td>
<td>46b</td>
<td>77b</td>
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<tr>
<td>TNF</td>
<td>57**†</td>
<td>63**†</td>
<td>55b</td>
<td>71b</td>
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<tr>
<td>Glutathione reductase (units/mg of protein)</td>
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<td></td>
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<tr>
<td>Ad libitum</td>
<td>5.8°</td>
<td>7.5c</td>
<td>9.3b°</td>
<td>10.1*</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Pair fed</td>
<td>4.1*b</td>
<td>4.9**b</td>
<td>5.0*b°</td>
<td>4.4**b</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>6.3†</td>
<td>9.5*</td>
<td>8.5°†</td>
<td>10.5†</td>
<td>0.3</td>
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<tr>
<td>γ-Glutamylcysteine synthetase (units/mg of protein)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Ad libitum</td>
<td>0.92</td>
<td>1.04</td>
<td>1.23</td>
<td>0.98</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>Pair fed</td>
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<td>1.21</td>
<td>1.39</td>
<td>0.11</td>
<td></td>
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<tr>
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<td>1.00</td>
<td>1.18</td>
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</tbody>
</table>
E. A. L. Hunter and R. F. Grimble

Table 7. Influence of cysteine and methionine supplementation on the action of endotoxin (LPS) on liver glutathione and glutathione synthesis of rats fed low-protein diets. Values are means, n = 4 per group. Within a row, values with different letter superscripts differ significantly (P < 0.05). *LPS or pair fed is different from ad libitum value (P < 0.05). †LPS is different from pair fed (P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Normal-protein diet</th>
<th>Low-protein diets</th>
<th>Cysteine</th>
<th>Methionine</th>
<th>Alanine</th>
<th>Pooled SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione (μmol/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>5.8b</td>
<td>6.5b</td>
<td>9.1b</td>
<td>4.6b</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Pair fed</td>
<td>3.8b**</td>
<td>2.8b**</td>
<td>3.3b**</td>
<td>2.7b**</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>5.5b†</td>
<td>6.5b†</td>
<td>7.5b†</td>
<td>3.5b†</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Glutathione synthesis rate (nmol min⁻¹ g⁻¹ of liver)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>19b</td>
<td>12b</td>
<td>39b</td>
<td>14b</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>Pair fed</td>
<td>9b</td>
<td>4*</td>
<td>9*</td>
<td>0*</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>15†</td>
<td>12*</td>
<td>17**</td>
<td>8*</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>Glutathione synthesized (μmol/day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>207b</td>
<td>132c</td>
<td>421c</td>
<td>90c</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Pair fed</td>
<td>72a*</td>
<td>31b</td>
<td>71b</td>
<td>0*</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>200a</td>
<td>117b</td>
<td>151b†</td>
<td>56b†</td>
<td>36</td>
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</tr>
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</table>

Differences in the response to food intake or an inflammatory stimulus were found between the dietary groups in both liver and lungs. In liver, when animals were consuming diets ad libitum, peroxidase activity was enhanced in the low-protein diets supplemented with alanine or methionine. A reduction in food intake per se had no effect on enzyme activity. Inflammatory challenge increased activities only in the normal-protein or low-protein group supplemented with cysteine. These responses indicate that a low cysteine intake or an inflammatory challenge in the presence of high cysteine intakes are prerequisites for enhanced peroxidase activity. All dietary groups receiving TNF had the same, elevated, liver glutathione peroxidase activity.

In contrast to the situation in liver, glutathione peroxidase in lung was mostly unaffected when diets were fed ad libitum. Furthermore, unlike in liver, restricted feeding caused major increases in the glutathione peroxidase activity of some dietary groups, namely in rats receiving low-protein diets supplemented with cysteine and alanine. Administration of TNF resulted in no further change in enzyme activity in these latter groups and increased activity in animals receiving the low-protein diets containing methionine. Consequently, all groups consuming low-protein diets had greater mean values for enzyme activity than in animals fed the normal-protein diet.

The pattern of response of glutathione reductase activity in liver (Table 6) to restricted feeding and the inflammatory stimulus was similar to glutathione concentration, in that pair feeding caused a reduced activity of the enzyme, compared with the ad libitum groups, while the inflammatory response to TNF prevented a fall in activity in all dietary groups. The effect of diet composition per se was different however. All animals consuming low-protein diets

Table 8. Influence of cysteine and methionine supplementation on the actions of TNF on lung glutathione and glutathione peroxidase and glutathione reductase activity of rats fed low-protein diets. Values are means, n = 5 per group. Within a row, values with different letter superscripts differ significantly (P < 0.05). **TNF or pair fed is different from ad libitum value (P < 0.05). †TNF is different from pair fed (P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Normal-protein diet</th>
<th>Low-protein diets</th>
<th>Cysteine</th>
<th>Methionine</th>
<th>Alanine</th>
<th>Pooled SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione (μmol/g)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>2.5b</td>
<td>2.6b</td>
<td>3.0b</td>
<td>2.0*</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Pair fed</td>
<td>2.0a*</td>
<td>2.0a*</td>
<td>2.2*</td>
<td>1.9b</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>TNF</td>
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<td>2.2b**</td>
<td>2.1b</td>
<td>1.9c</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase (units/g of protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>36b</td>
<td>39b</td>
<td>53b</td>
<td>38b</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Pair fed</td>
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<td>68a*</td>
<td>44b</td>
<td>61a**</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>41b</td>
<td>62a**</td>
<td>71a**</td>
<td>73a*</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Glutathione reductase (units/mg of protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>6.8b</td>
<td>8.6b</td>
<td>10.8b</td>
<td>7.7b</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Pair fed</td>
<td>10.0b**</td>
<td>13.2a**</td>
<td>9.7b</td>
<td>13.1a*</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>7.3†</td>
<td>8.6b</td>
<td>9.6b</td>
<td>8.9†</td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>
Glutathione metabolism during inflammation

ad libitum had higher mean values for reductase activity than animals fed the normal-protein diet. Values were highest when the diet was supplemented with alanine. A value 174% of that found in the group receiving the normal-protein diet was observed. There were similarities and differences between the responses in liver and lung. In lung, higher mean values for glutathione reductase were observed in all groups fed low-protein diets ad libitum, compared with values in animals fed the normal-protein diet. Values were only significantly elevated when the low-protein diet was supplemented with methionine. Under these conditions a value which was 159% of the value in the normal-protein group was observed. Food restriction caused large increases in activity in all groups except for rats receiving the methionine-supplemented diet. The inflammatory challenge of TNF resulted in similar values to those found when each group was receiving diets ad libitum. Thus with the exception of rats fed the low-protein diet containing methionine, food restriction and the inflammatory challenge produced reciprocal changes in the activity of glutathione reductase in lung and liver. No effect of diet, food restriction or TNF on γ-glutamylcysteine synthetase activity was found (Table 6).

Hepatic glutathione synthesis

The values for hepatic GSH synthesis are shown in Table 7. Various methods have been used to estimate the rate of synthesis of glutathione in liver. All show a rate of approximately 20 nmol min⁻¹ g⁻¹ in rats consuming diets of an adequate protein content [17, 23, 26]. Values in the present study were of similar magnitude.

In animals fed ad libitum, the highest rates of hepatic GSH synthesis occurred in rats receiving the low-protein diet supplemented with methionine (Table 7). Animals receiving the other low-protein diets ad libitum had mean values for synthesis rates that were lower than those observed in rats receiving the normal-protein diet, however, the differences did not reach statistical significance. Restricted feeding caused a reduction in glutathione synthesis in all dietary groups, the fall effectively halting glutathione synthesis in the animals fed the low-protein diet supplemented with alanine. LPS treatment prevented the fall in glutathione synthesis due to the reduced food intake. However, in the methionine-supplemented animals, the rate of synthesis was not returned to values seen in animals fed ad libitum. As a result there were no significant differences between the values in the various dietary groups when LPS was given. However, the lowest mean value was found in animals receiving low-protein diets supplemented with alanine. A similar pattern of responses occurred when glutathione synthesis was expressed as the amount synthesized per day (the product of liver weight and rate of synthesis, Table 7). However, after LPS treatment the mean value for rats fed the low-protein diet supplemented with alanine was significantly lower than for animals fed the normal-protein diet and the low-protein diet supplemented with methionine.

DISCUSSION

The important role that glutathione plays during the response to inflammatory agents and xenobiotics has been reported by many authors [2, 4]. In studies in which rats were stressed with ozone, an initial fall in glutathione concentration, followed by an increase in both liver and lung glutathione, was found, demonstrating the possibility that GSH is being replaced by either reduction of oxidized glutathione to GSH or de novo synthesis of glutathione in liver, and by the former mechanism in lung [27, 28]. In addition, increased uptake of GSH from the bloodstream provides an additional means of maintaining the concentration in lung. The results in the present study indicate that these methods of maintaining or enhancing tissue GSH content occur and are influenced by dietary sulphur amino acid adequacy.

In the absence of an inflammatory response, glutathione concentrations show a predictable response to changes in total sulphur amino acid and food intakes. When diets were consumed ad libitum, the low-protein diet supplemented with alanine resulted in the lowest value for hepatic and lung GSH. Addition of sulphur amino acids to such diets raised GSH concentrations but resulted in higher values after dietary supplementation with methionine than with cysteine. This phenomenon is in contrast with studies which show glutathione synthesis to be more sensitive to cysteine than methionine supply in the medium of cultured hepatocytes or in the diets of rats [29, 30]. The consequence of a restriction of food intake (and hence further reduction in sulphur amino acid intake) observed in the pair-fed groups was a reduction in GSH concentration in both tissues. However the magnitude of change was greater in the liver than in lung. The inflammatory challenge overcame the influence of appetite loss and restored tissue GSH towards values observed in animals fed ad libitum, in a manner which is modulated by the dietary sulphur amino acid and protein intake before the challenge. In animals fed diets of adequate sulphur amino acid content, liver glutathione concentrations were elevated compared with their pair-fed counterparts. This increase was not observed in the alanine-supplemented group. Similar observations have been reported previously [11, 16]. The present study indicates that lung GSH concentrations are sensitive to food and sulphur amino acid intake in a similar way to the concentration in liver. These findings are in agreement with observations from other studies [28, 31, 32].
However the concentration in lung was restored to
normal by an inflammatory stimulus only in animals
fed the normal-protein diet.

Absolute and relative changes in the activities of
 glutathione peroxidase and reductase may have a
 bearing on the concentration of GSH within tissues.
 However activities of the reductase in the present
 studies are at least 57-fold greater than that of
 peroxidase in liver (low-protein alanine group, pair
 fed) and 121-fold greater in lung (low-protein
 alanine group, TNF treated). This relative balance
 of activity of the two enzymes will facilitate the
 return of cellular glutathione to the reduced state.
 Thus changes in the activity of reductase, which
 occurred in the present study as a result of dietary
 change or inflammation, have a greater potential
 impact on recycling of GSH than alterations in
 peroxidase. Changes in peroxidase activities in liver
 and lung of the TNF-treated groups compared with
 the animals fed ad libitum indicate increased utiliza-
 tion of GSH during an inflammatory response,
 either as a consequence of production of oxidant
 molecules in response to the cytokine, or in liver
 because of increased utilization of GSH in the
 metabolism of xenobiotics [2]. However, the latter is
 less likely since TNF decreases the activity of
 cytochrome P-450, a key enzyme in xenobiotic
 metabolism [33]. Reductase activity is clearly sensi-
 tive to dietary protein and sulphur amino acid
 intake. Consumption of the low-protein diet ad
 libitum resulted in a general increase in reductase
 activity in liver and lung. In liver, the highest values
 were observed in animals consuming diets with low
 cysteine contents (80 g/kg of casein supplemented
 with methionine or alanine). Dietary energy restric-
 tion reduced reductase activity in the livers of all
dietary groups. A similar effect was observed by
 Bauman et al. [1]. In lung, reductase activity was
 greatly increased by food restriction. Thus while the
 recycling of GSSG to GSH may have been facilitat-
ed in liver and lung when protein intake was
 suboptimal, when the suboptimal intake was
 combined with reduced energy intake, the effect was
 enhanced in lung but suppressed in liver. The
 response in liver to a low protein and cysteine intake
 is reversed by an inflammatory challenge, and would
 result in an increased capacity to recycle GSSG to
 GSH in liver, thereby helping to increase and
 maintain GSH concentrations. In lung, however,
 TNF restored the elevated reductase levels, which
 resulted from low food intake, to values seen in
 animals consuming the diets ad libitum. The effect
 of TNF on lung reductase activity in the rat
 contrasts with the effects of an inflammatory
 stimulus, in the form of endotoxin, in mouse. In this
 species, an inflammatory stimulus increased glu-
 thione reductase activity in lung [34]. No significant
 differences in the activity of γ-glutamylcysteine
 synthetase, the rate-limiting enzyme of glutathione
 synthesis, occurred as a result of a reduction in
 sulphur amino acid intake or the inflammatory
 challenge. However, the lack of effect of diet or
 inflammatory stimuli on the enzyme does not
 preclude changes in glutathione synthesis, since
 measurement of γ-glutamylcysteine synthetase
 activity indicates the capacity of the tissue for
 synthesis and does not consider the flow of available
 substrate through the enzymic reaction in vivo.

Our previous studies [9, 11, 14] have suggested
 that the sulphur amino acids play an important role
 in the inflammatory response by acting as substrates
 for glutathione and acute-phase protein synthesis.
 Since cysteine is also required for components of
 the inflammatory response, such as for lymphocyte
 function and synthesis of metallothionein and other
 proteins [4, 9, 35], then in situations where cysteine
 availability is reduced, such as occurred with the
 low-protein diets supplemented with alanine, the
 competing demand for cysteine may result in
 reduced incorporation into GSH. Indeed studies in
 vitro have shown that cysteine (the dipeptide of
 cysteine) is the rate-limiting precursor for the
 synthesis of GSH by human fibroblasts and mouse
 macrophages [36, 37]. Reeds and colleagues
 questioned the importance of cysteine in the
 synthesis of protein during an acute-phase response,
 due to the amino acid composition of some of the
 key acute-phase proteins, although the requirement
 of cysteine for glutathione was described as being
 'probably of importance' [38]. While quantitatively
 more of the aromatic amino acids are required for
 synthesis into hepatic protein during the inflamma-
tory response, compared with cysteine [38], the
 importance of cysteine in enabling glutathione
 synthesis during an inflammatory response must not
 be overlooked [4, 6, 9]. In a previous study, we
 measured the rates of synthesis of tissue protein in
 rats injected with TNF. The animals were of similar
 age, sex and strain to those in the present study and
 were fed the diets used in the present study [11]. In
 animals fed normal-protein diets, total hepatic rates
 of protein synthesis increased from 0.96 g/day to
 1.48 g/day, after TNF injection. Such rates represent
 incorporation of cysteine into protein of 128 and
 197 μmol/day, based on a cysteine content of liver
 protein of 133.2 μmol/g of protein [13]. In the
 present study, the calculated rates of glutathione
 synthesis represent an incorporation rate of cysteine
 of 76 and 200 μmol/day in pair-fed and LPS-treated
 animals, of the normal-protein group, respectively.

Thus it appears that cysteine may be incorporated
 into hepatic glutathione with a similar magnitude to
 incorporation into protein when diets containing
 adequate amounts of sulphur amino acids are fed.
 Indeed, using the earlier assumptions, for animals
 fed the low-protein diets supplemented with alanine,
 incorporation of cysteine into hepatic glutathione
 would be 0 and 56 μmol/day in the pair-fed and LPS
 groups respectively. In our previous study, incor-
 poration of cysteine into total hepatic protein, for
 animals fed a similar diet, would be at the rates of
 48 and 139 μmol/day for pair-fed and TNF-treated
animals respectively [11]. Thus the ability to synthesize GSH may be compromised to a much greater degree than the ability to synthesize protein during dietary sulphur amino acid insufficiency. Indeed, the characteristics of the enzymes which are rate limiting for the incorporation of cysteine into protein and glutathione (L-cysteine-tRNA synthetase E.C. 6.1.1.16 and γ-glutamylcysteine synthetase respectively) favour this metabolic outcome when cellular concentrations of cysteine are low, since they have K_m values in the micromolar and millimolar range respectively [39, 40]. Such a situation may lead to antioxidant defences becoming compromised and may contribute to the reduced cellular GSH concentrations observed in individuals infected with HIV [6].

ACKNOWLEDGEMENTS

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REFERENCES

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