Glutathione Turnover Is Increased during the Acute Phase of Sepsis in Rats[1,2]

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ABSTRACT Glutathione metabolism during infection has been poorly documented. Glutathione concentrations and synthesis rates were studied in infected rats (2 d after infection) and in pair-fed controls. Glutathione synthesis rates were determined in liver, spleen, lung, small and large intestine, skeletal muscle, heart and blood by a 4-h or 6-h ^{15}N cysteine infusion. The activities of four hepatic enzymes involved in glutathione metabolism were also determined. Glutathione synthesis rates were significantly greater in liver (+465%), spleen (+388%), large intestine (+109%), lung (+100%), muscle (+91%) and heart (+80%) of infected rats compared with pair-fed controls. Glutathione concentrations were also greater in these tissues but were unaffected in small intestine and lower in blood. In keeping with the stimulation of liver glutathione synthesis, the activities of liver γ-glutamyl-cysteine synthetase and glutathione reductase were significantly greater in liver of infected rats than of pair-fed rats. From the present study, we estimate that glutathione synthesis accounts for at least 40% of the enhanced cysteine utilization during infection. This increased utilization may be the primary cause of an enhanced cysteine requirement in infection. J. Nutr. 130: 1239–1246, 2000.

KEY WORDS: • *rats* • *glutathione synthesis rate* • *cysteine infusion* • *glutathione-related enzymes*

Reduced glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH)[4] is a tripeptide that is present in nearly all animal cells and is the predominant intracellular low-molecular-weight thiol compound. GSH has many important roles in the cell (Beutler 1989, Deneke et al. 1985). It is involved in DNA and protein synthesis (Kan et al. 1988, Suthanthiran et al. 1990) and the synthesis of leukotrienes, which are important mediators of inflammation (Rouzer et al. 1982). GSH also maintains the reduced state of the sulfhydryl groups of many proteins, which is required for their normal function (Gérard-Monnier and Chaudière 1996). It acts as a cysteine reserve during food deprivation (Cho et al. 1981) and is a major source of cysteine for lymphocytes. Indeed, the normal activity of lymphocytes is dependent on cellular availability of this sulfur amino acid (Dröge et al. 1986). GSH is also very important in the protection of cells against toxic insult (Beutler 1989, Deneke et al. 1985). It participates in the detoxification of electrophilic metabolites of xenobiotics and is a very efficient free radical scavenger, protecting cells from the toxic effects of reactive oxygen compounds (Beutler 1989). During injury, oxidative stress is increased (Fagan et al. 1996, Peralta et al. 1993). This stress is of prime importance for the organism's defense but may have deleterious effects on tissues if it is not tightly controlled by antioxidants. This can be seen very clearly in GSH-depleted rats where hyperoxia induces an increased mortality rate (Deneke et al. 1985).

GSH is synthesized by nearly all organs and tissue beds in a two-step metabolic pathway. The first step, conversion of glutamic acid and cysteine in γ-glutamyl-cysteine, is catalyzed by the enzyme γ-glutamyl-cysteine synthetase. This step is rate-limiting and is feed-back inhibited by GSH. The second step is the conversion of γ-glutamyl-cysteine to GSH. Numerous other enzymes participate in GSH metabolism. Glutathione peroxidase catalyzes the formation of oxidized glutathione (GSSG) during the reduction of hydroperoxides. GSH can be regenerated from GSSG by GSSG reductase. During the detoxification processes, toxic compounds are attached to GSH by glutathione transferase. This is followed by further reactions which result in a net loss of glutathione. The glutathione status of cells is therefore dependent on the relative activity of these reactions. Indeed, increased activity of enzymes involved in GSH synthesis and of GSH reduction can lead to an increased GSH concentration. Conversely, increased activity of GSH peroxidase and GSH transferase decreases GSH concentration.

The glutathione level is altered in many inflammatory conditions. A fall in GSH concentration has been reported in human diseases such as HIV infection and trauma (Luo et al. 1998, Staal et al. 1992). In animal models, during the initial

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[4] Abbreviations used: APE, atom % excess; ASR, absolute synthesis rate; CDNB, 1-chloro-2,4-dinitrobenzene; GSH, glutathione; GSSG, oxidized glutathione; ks, synthesis rate; TNF, tumor necrosis factor.

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phase of septic shock (during the first hours after infection), tissue GSH concentrations are decreased whereas GSSG concentrations are increased (Ikegami et al. 1994, Keller et al. 1985). In contrast, during the first days following infection, tissue glutathione levels are elevated in treated animals compared with controls (Hunter and Grimble 1997, Malmezat et al. 1998). However, in the later stages of infection, depletion of tissue GSH levels occurs (Breuillé et al. 1994a, Colomb et al. 1995).

Variations in glutathione levels during oxidative conditions may result from modification in synthesis and/or loss. To determine the relative importance of these two potential mechanisms, it is necessary to measure glutathione kinetics in vivo. However, mechanisms responsible for maintenance of GSH homeostasis in different tissues are poorly documented, especially in pathological conditions. The aim of this study was to measure glutathione concentrations, glutathione synthesis rates and the activities of several enzymes involved in glutathione metabolism in various tissues of infected rats. Infection induces a strong anorexia. Because glutathione concentrations are decreased by food deprivation (Hunter and Grimble 1997, Jahoor et al. 1995), pair-fed rats were used as controls.

MATERIALS AND METHODS

Animals and experimental design. Male Sprague-Dawley rats (Iffa-Credo, Lyon, France), 270–280 g body weight, were individually housed in wire-bottom cages and consumed ad libitum a semisynthetic diet containing 12 g protein/100 g diet (Malmezat et al. 1998). After an acclimation period of 5 d, rats in the post-absorptive state were anesthetized with ketamine (50 mg/kg; Imalgene®, Rhône Mérieux, Lyon, France) and acepromazine (2.5 mg/kg; Vetranquil®, Sanofi, Libourne, France). Using sterile techniques, silicone catheter (Silastic tubing; Sigma Medical, Nanterre, France) was inserted into the right jugular vein and flushed with heparinized normal saline. The free-end of the catheter was tunnelled subcutaneously and exteriorized dorsally on the head, through a flexible spring secured to the top of the head with dental cement. The infusion line passed through the spring and was connected to a swivel suspended from the top of the cage, which allowed free movement of the rat. During a 10-d recovery period, rats were continuously infused with saline at 0.1 mL/h. Animals grew ~6 g/d during the recovery period.

The rats were then injected via a tail vein, with either live *Escherichia coli* (6.6×10^8 bacteria per rat, infected group, $n = 13$) or saline (control group, $n = 8$) as described previously (Malmezat et al. 1998). Since infection induces a strong anorexia, control rats were pair-fed the mean intake of the infected rats.

Forty-eight hours after injection of bacteria or saline, food was withdrawn. A primed-continuous infusion of ^{15}N cysteine (Cambridge Isotope Laboratories, Andover, MA) into the jugular vein was started. The priming dose was 1.5 μmol/100 g, and the isotope was continuously infused at 2.4 μmol/(100 g · h), for 4 h in five infected rats (Group 1) and for 6 h in eight infected rats (Group 2). Blood samples were taken from a tail vein at 30 min, 1, 2 and 4 h after the beginning of the infusion in Group 1, and 5 h and 5.5 h after the beginning of the infusion in Group 2. Pair-fed rats were fed a priming dose of 1.1 μmol/100 g and 6 h infusion of ^{15}N cysteine at a rate of 1.8 μmol/(100 g · h). Different priming dose and infusion rates were used in pair-fed rats and in infected rats to obtain the same cysteine enrichment in free amino acid pool in both groups. Blood samples were taken from a tail vein 5 h and 5.5 h after the start of the infusion. At the end of the infusion, rats were anesthesized (pentobarbital, 60 mg/kg). Blood was withdrawn from the vena cava, and liver, spleen, lungs, heart, small and large intestine and muscles from the hind leg were rapidly excised. Liver, spleen, heart and digestive tract were flushed and all tissues frozen in liquid nitrogen and kept at −80°C until analysis.

The protocol was approved by the Ethics Committee of the Institute and conducted in conformity with the guiding principles in the care and use of laboratory animals.

Analytical procedures. Tissues were finely pulverized in liquid nitrogen in a ball mill (Dangoumeau; Prolabo, Paris, France). An aliquot of frozen powder was homogenized by sonication in 0.2 mol/L perchloric acid containing 5 mmol/L EDTA. The homogenate was then centrifuged (20 min, 8000 × *g*), and the supernatant assayed for glutathione content, using a standard enzymatic recycling procedure as described previously (Malmezat et al. 1998). Blood glutathione has been measured by the same method on 10% (v/v) hemolysates prepared in water.

For enzymatic activity measurements in liver, frozen powders were homogenized in phosphate buffer (phosphate 75 mmol/L, dithiotreitol 2 mmol/L, sucrose 0.3 mol/L). Homogenates were centrifuged (1 h, 100,000 × *g*), and enzymatic activities were measured in the supernatants. Glutathione reductase (EC 1.6.4.2) was measured in the presence of GSSG by following the oxidation of NADPH at 340 nm (Carlberg and Mannervik 1975). Glutathione peroxidase (EC 1.11.1.9) was measured in the presence of GSH and H_2O_2 by following the disappearance of NADPH at 340 nm (Paglia and Valentine 1967). The activity of γ-glutamyl-cysteine synthetase (EC 6.3.2.2) was measured by the coupled enzyme assay of Seelig and Meister (1985), using pyruvate kinase and lactate dehydrogenase. Glutathione *S*-transferase (EC 2.5.1.18) was measured in the presence of GSH and 1-chloro-2,4-dinitrobenzene (CDNB) by following the formation of GSH-CDNB at 340 nm (Habig et al. 1974). Liver protein concentration was determined using the bicinchoninic acid procedure (Pierce Chemical Co., Rockford, IL) (Smith et al. 1985).

Determination of free and glutathione-bound ^{15}N cysteine enrichment. Free and glutathione-bound ^{15}N cysteine enrichments were measured on their *N,S* ethoxyl carbonyl methyl esters, using a method adapted from Kataoka et al. (1995). An aliquot of plasma or blood was diluted (1/2) in a 0.2 mol/L phosphate buffer (EDTA 5 mmol/L, dithiotreitol 80 mmol/L, pH 8.5). Aliquots of powdered frozen tissues were homogenized by sonication in the same phosphate buffer. The mixture was left at room temperature for 15 min and then the pH was adjusted to between 7 and 8. The *N,S*-ethoxycarbonyl methyl esters of cysteine and glutathione were prepared as follows. Ethyl chloroformate (0.1 mL) (Acros Organics, Geel, Belgium) was added to the sample and mixed by rotation at 100 rpm for 15 min at room temperature (tube rotator; Poly Labo, Strasbourg, France). The reaction mixture was then acidified to pH 1–2 with 2 mol/L HCl, saturated with NaCl and extracted twice with 3 mL of peroxide-free diethyl ether. The ether extracts were evaporated to dryness at 80°C, and then 0.2 mL of 1 mol/L HCl in methanol was added to the residue and the mixture was incubated at 80°C for 10 min. The residual solvent was evaporated to dryness at 80°C under a stream of air and the residue was finally dissolved in 0.1 mL of ethyl acetate. Free- and glutathione-bound cysteine enrichments were then measured by gas chromatography-mass spectrometry, with an HP 5890 gas chromatograph coupled with a HP 5972 organic mass spectrometer quadripole (Hewlett-Packard, Les Ulis, France). Gas chromatograph analysis was conducted on a crosslinked 5% diphenyl 95% dimethyl siloxane capillary column (0.25 mm i.d. × 0.25 mm film thickness) (HP S M 5; Hewlett-Packard). The length of the column was 30 m for cysteine and 10 m for glutathione. Free ^{15}N cysteine and ^{14}N cysteine were monitored at $m/z = 221$ $[M + 1 - 59]^-$ and at $m/z = 220$ $[M - 59]^-$, respectively. Glutathione-bound ^{15}N cysteine and glutathione-bound ^{14}N cysteine were monitored at $m/z = 364$ $[M + 1 - 116]^-$ and at $m/z = 363$ $[M - 116]^-$, respectively.

Calculations. The fractional synthesis rate (ks) of tissue glutathione was calculated according to the precursor-product relationship. When a labeled amino acid such as ^{15}N cysteine is given by constant infusion, the isotopic enrichment (atom % excess, APE) of the precursor pool reaches a constant value with time. By measuring the enrichment of the labeled amino acid in the product (glutathione) when there is a steady state in the precursor pool, ks can be obtained using the following equation:

$$dEbt/dt = ks \cdot Ef \quad (1)$$

where dEbt is the increase in isotopic enrichment of reduced gluta-

thione-bound cysteine in tissues over the period dt, and Ef is the isotopic enrichment of free cysteine in tissues.

Ef is not constant during the entire infusion period and the plateau value (Ef max) is not reached immediately. However, the time course of Ef can be described by a simple exponential of the form (Waterlow et al. 1978):

$$Ef = Ef\ max\ (1 - e^{-\lambda t}) \quad (2)$$

where Ef max is tracer/tracee ratio during the steady state and λ is an empirically-fitted rate constant.

The time course of the enrichment of free cysteine in plasma can also be described by a simple exponential analogous to the equation presented above for tissues. If we assume that the value of λ in tissues can be taken as equal to the value determined in plasma (Waterlow et al. 1978), equation 1 becomes:

$$dEbt/dt = ks \cdot Ef\ max\ (1 - e^{-\lambda t})$$

and after integration,

$$Ebt/Ef\ max = ks \cdot [t\lambda - (1 - e^{-\lambda t})]/\lambda$$

then

$$ks = (Ebt/Ef\ max) \cdot \lambda/[t\lambda - (1 - e^{-\lambda t})]$$

In a previous study we found that the value of λ was similar in infected and pair-fed rats.

The absolute synthesis rate (ASR) of tissue reduced glutathione was calculated as the product of tissue reduced glutathione concentration and ks.

The standard steady-state equation was used to calculate plasma cysteine flux in the circulation as follows:

$$Cysteine\ flux = I \times (Etr/Epl - 1)$$

where I is the infusion rate of ^{15}N cysteine, Etr the enrichment of cysteine tracer and Epl the enrichment of plasma cysteine at plateau.

Statistics. Values are given as means ± SD. The statistical significance of differences between means was assessed by one-way ANOVA. Unpaired Student's *t* tests were used to compare data between the two groups of infected rats and between infected and pair-fed rats. Differences were accepted as significant when $P < 0.05$.

RESULTS

Food consumption, rat body and tissue weights. Before infection, rats consumed about 26 g of dry matter per day. On the day of infection, food was mainly consumed in the morning, before infection (4.4 ± 3.9 g). One day after infection, the rats ate 10.6 ± 5.7 g, i.e., about 60% less than before injection of bacteria.

Food-intake restriction produced a body weight loss in pair-fed rats. However, infected rats lost significantly more weight than pair-fed controls (**Fig. 1**).

Weights of tissues are expressed as g/300 g body weight at d 0. Weights of lung, heart and large intestine did not differ between the two groups. By contrast, relative weights of liver, spleen and small intestine were 58, 167 and 11% ($P < 0.05$) significantly greater, respectively, in infected rats than in pair-fed rats Gastrocnemius weight was significantly reduced 31% in septic rats compared with pair-fed rats (**Table 1**).

Glutathione concentration. Total glutathione concentration was significantly greater in septic rats than in pair-fed controls in all tissues except small intestine and blood (**Table 2**). Because liver and spleen weights were also greater in infected rats than in pair-fed rats, the total amount of glutathione in these two tissues in the septic rats was more than double that of the pair-fed rats. There was no difference in small intestine glutathione concentration between the two groups, whereas blood glutathione concentrations were significantly lower in infected rats than in pair-fed rats (Table 2).

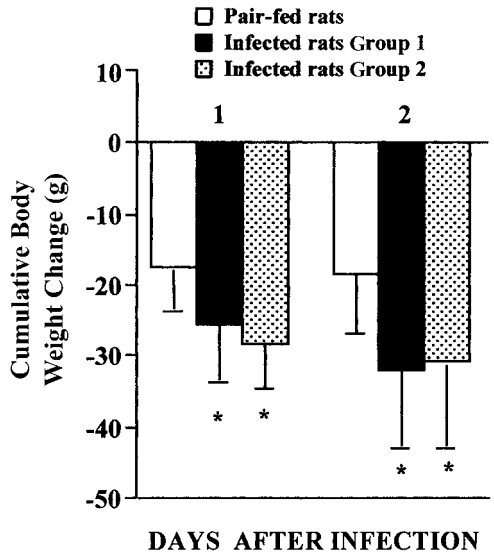

FIGURE 1 Effect of infection on cumulative body weight change. Two groups of rats were infected by an intravenous injection of live *Escherichia coli* ($6.5 \cdot 10^8$). One control pair-fed group was injected intravenously with saline and ate the same amount of food as the infected rats. Values are means ± SD for infected rats (Group 1, $n = 5$; group 2, $n = 8$) and pair-fed rats ($n = 8$). *Body weight losses of infected rats Groups 1 and 2 were significantly lower than that of pair-fed rats.

In control rats consuming food ad libitum (Breuillé et al. 1994a) liver and spleen glutathione concentrations (5.18 ± 0.42 μmol/g liver and 2.82 ± 0.19 μmol/g spleen) were significantly greater than in pair-fed rats and significantly lower than in infected rats. By contrast, both infection and food restriction induced a fall in gastrocnemius glutathione concentration when compared to glutathione concentration values in control rats (0.91 ± 0.12 μmol/g). In the total digestive tract, glutathione concentration in control rats (2.46 ± 0.26 μmol/g) was significantly greater than in pair-fed rats (2.22 ± 0.16 μmol/g) and not different from infected rats (2.44 ± 0.19 μmol/g).

TABLE 1

Body and relative tissue weights of infected and pair-fed control rats at d 0[1]

	Pair-fed rats	Infected rats[2]
	g/300 g body	
Body	270 ± 14	274 ± 18
Liver[3]	7.6 ± 0.3	12.0 ± 1.2*
Spleen	0.6 ± 0.1	1.7 ± 0.3*
Gastrocnemius	1.8 ± 0.2	1.2 ± 0.5*
Lung	1.4 ± 0.1	1.5 ± 0.2
Heart	0.9 ± 0.1	1.0 ± 0.1
Small intestine	5.3 ± 0.4	5.9 ± 0.5*
Large intestine	2.2 ± 0.1	2.0 ± 0.2

* Significantly different from pair-fed rats, $P < 0.05$.

[1] Mean values ± SD are given for eight rats in each group (infected rats, group 2)

[2] Two groups of rats were infected by an intravenous injection of live *Escherichia coli* ($6.5 \cdot 10^8$). One pair-fed control group was injected intravenously with saline and ate the same amount of food as the infected rats. Measurements were made 2 d after infection.

[3] Liver values were obtained in infected rats of Group 1, $n = 5$.

TABLE 2

Total glutathione concentrations in tissues of infected and pair-fed control rats[1]

Tissues	Pair-fed rats	Infected rats
	μmol/g tissue	
Liver[2]	3.52 ± 0.21	8.18 ± 1.40*
Spleen	2.45 ± 0.09	3.22 ± 0.2*
Gastrocnemius	0.56 ± 0.13	0.78 ± 0.11*
Lung	1.81 ± 0.09	2.01 ± 0.11*
Heart	1.53 ± 0.11	2.02 ± 0.14*
Small intestine	2.45 ± 0.21	2.61 ± 0.21
Large intestine	1.65 ± 0.14	1.94 ± 0.18*
Blood	1.60 ± 0.09	0.79 ± 0.21*

* Significantly different from pair-fed rats, $P < 0.05$.

[1] Two groups of rats were infected by an intravenous injection of live *Escherichia coli* ($6.5 \cdot 10^8$). One pair-fed control group was injected intravenously with saline and ate the same amount of food as the infected rats. Measurements were made 2 d after infection. Mean values ± SD are given for eight animals in pair-fed and infected rats Group 2 (except for values obtained in liver where $n = 5$ for infected rats Group 1).

[2] Glutathione liver concentration was measured in Group 1 for infected rats. Value obtained in group 2 (6.86 ± 1.52 μmol/g liver) was not significantly different than values obtained in Group 1.

Activities of enzymes involved in glutathione metabolism. The specific activity of γ-glutamylcysteine synthetase was not significantly different between the infected group and the pair-fed group (**Table 3**). In contrast, since liver weight and protein content were significantly greater in infected rats, the total activity of γ-glutamyl-cysteine synthetase was 50% greater in infected compared to pair-fed rats (Table 3). The hepatic activity of glutathione reductase was significantly higher in infected compared to pair-fed rats, when expressed as specific (16% increase), or total activity (60% increase) (Table 3).

TABLE 3

Specific and total activity of the enzymes linked to glutathione metabolism in liver of infected and control pair-fed rats[1]

	γ Glutamyl-cysteine synthetase	Glutathione reductase	Glutathione peroxidase	Glutathione transferase
		units/g protein		
Pair-fed rats	80.6 ± 8.1	40.9 ± 4.2	742 ± 133	535 ± 48
Infected rats	88.0 ± 15.4	47.5 ± 4.9*	604 ± 92*	482 ± 75
		units/liver		
Pair-fed rats	123 ± 18	62.1 ± 6.8	1129 ± 233	815 ± 104
Infected rats	184 ± 31*	98.9 ± 8.1*	1255 ± 108	999 ± 95*

* Significantly different from pair-fed rats, $P < 0.05$.

[1] Rats were infected by an intravenous injection of live *Escherichia coli* ($6.5 \cdot 10^8$). Pair-fed control rats were injected intravenously with saline and ate the same amount of food as the infected rats. Measurements were made 2 d after infection. Mean values ± SD are given for eight animals in pair-fed and infected rats Group 2.

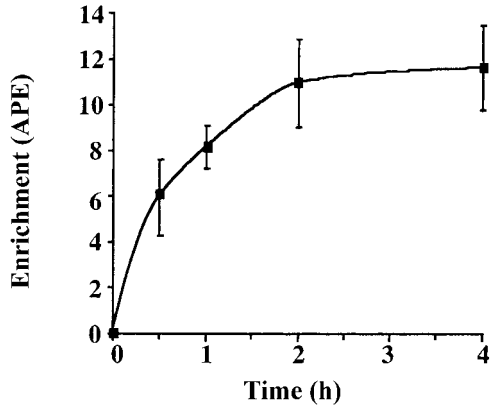

FIGURE 2 Enrichment of plasma cysteine during a 4-h infusion of ^{15}N cysteine in infected rats (Group 1). Rats were infected by an intravenous injection of live *Escherichia coli* ($6.5 \cdot 10^8$). Two days after infection, the rats were given a primed constant intravenous infusion of ^{15}N cysteine. Each data point represents the means ± SD of five rats. APE, atom% excess.

The GSH peroxidase specific activity was lower in the infected group compared to the pair-fed group, but the total activity did not differ between groups (Table 3). The specific activity of GSH transferase was also not different in two groups of rats, but its total activity was 23% greater ($P < 0.05$) in the infected compared to the pair-fed rats (Table 3).

Cysteine and glutathione kinetics. The empirically fitted rate constant λ for cysteine was determined from serial samples of blood taken in infected rats from Group 2 (**Fig. 2**) and was estimated to be 30 d^{-1}.

The enrichment (APE) of free plasma cysteine reached a steady state after 2 h of isotope infusion (Fig. 2). This steady state was maintained until the end of the infusion in infected and pair-fed rats (**Fig. 3**). We assume that steady state was reached also in the liver, since the ratio of liver to plasma enrichment was not significantly different in infected rats at 4 h (Group 1) and 6 h (Group 2) of infusion (0.25 ± 0.04 and 0.28 ± 0.04, respectively, **Table 4**).

^{15}N cysteine enrichment in liver glutathione of infected

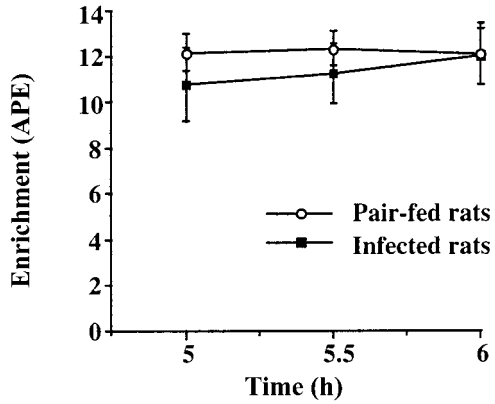

FIGURE 3 Enrichment of plasma cysteine during the last hour of the infusion of ^{15}N cysteine in infected (Group 2) and pair-fed rats. Rats were infected by an intravenous injection of live *Escherichia coli* ($6.5 \cdot 10^8$). The control pair-fed group was injected intravenously with saline and ate the same amount of food as the infected rats. Two days after infection, infected and pair-fed rats were given a primed constant intravenous infusion of ^{15}N cysteine for 6 h. Each data point represents the means ± SD of eight rats. APE, atom% excess.

TABLE 4

^{15}N enrichments of plasma and liver free cysteine, and liver glutathione-bound cysteine in infected and pair-fed control rats[1]

	Cysteine		Glutathione	
	Plasma enrichment	Liver enrichment	Liver enrichment	Liver ks
	APE			*%/d*
Pair-fed rats	12.1 ± 0.9	4.63 ± 1.34	3.91 ± 1.19	385 ± 33
Infected rats—Group 1	11.7 ± 1.6	2.97 ± 0.31	2.30 ± 0.24	534 ± 117*
—Group 2	12.2 ± 1.9	3.57 ± 0.78	3.47 ± 0.87	450 ± 66*†

* Significantly different from pair-fed rats. $P < 0.05$.

† Significantly different from infected rats Group 1. $P < 0.05$. ks, synthesis rate.

[1] Two groups of rats were infected by an intravenous injection of live *Escherichia coli* ($6.5 \cdot 10^8$). One pair-fed control group was injected intravenously with saline and ate the same amount of food as the infected rats. Two days after infection, rats were given a primed constant intravenous infusion of ^{15}N cysteine for 4 h (infected rats Group 1) or 6 h (infected rats Group 2 and pair-fed rats). Mean values ± SD are given for five rats in infected animals of Group 1 and eight in infected rats Group 2 and pair-fed rats group. APE: Atom % excess. Free and glutathione-bound ^{15}N cysteine enrichments were measured at the end of infusion in each group.

rats infused for 6 h (Group 2) was close to liver free cysteine enrichment in the same rats, suggesting that glutathione reached a plateau after 6 h of infusion (Table 4). It is likely that the glutathione synthesis rate calculated in these rats was probably underestimated, as illustrated by the values obtained in the infected rats infused for 4 h (Group 1). Nevertheless, the value obtained in infected rats infused for 6 h (Group 2) was significantly greater than in pair-fed rats.

Glutathione ks was significantly enhanced in liver (+39%), spleen (+37%), muscle (+68%) lungs (+49%) and large intestine (+95%) of septic rats compared to pair-fed rats (**Table 5**). In heart, there was a trend ($P < 0.09$) for a greater glutathione ks in septic rats than in pair-fed rats. Infection had no effect on glutathione ks in small intestine and blood (Table 5).

Because glutathione concentrations in numerous tissues were greater in infected rats than in pair-fed rats, ASR also were significantly enhanced in liver (+465%), spleen (+388%), muscle (91%), lungs (+100%), heart (+80%) and large intestine (+109%) in the former vs. latter groups (Table 5). The ASR for glutathione in the small intestine did not significantly differ in the two groups (Table 3). Blood glutathione ASR was significantly lower in septic rats than in pair-fed rats (−47%) (Table 5).

Cysteine flux was significantly greater in infected rats [18.3 ± 2.2 μmol/(h · 100 g body)] than in pair-fed rats [12.3 ± 1.7 μmol/(h · 100 g body)].

DISCUSSION

Fluctuations in tissue glutathione concentrations, as with any metabolite, depend on the balance between its ks and uptake from the circulation and the rate of its disappearance (which corresponds to catabolism and release into the circulation). To understand mechanisms responsible for the maintenance or depletion of glutathione in tissue, it is therefore necessary to measure the ks, uptake, catabolism and release of

TABLE 5

Glutathione synthesis rates in tissues of infected and pair-fed control rats[1]

	Fractional synthesis rate (ks)		Absolute synthesis rate (ASR)[2]	
Tissues	Pair-fed rats	Infected rats	Pair-fed rats	Infected rats
	%/d		*μmol/(tissue · d)*	
Liver	385 ± 33	534 ± 117*	89.5 ± 12.0	552 ± 214*
Spleen	249 ± 46	342 ± 36*	3.3 ± 1.6	18.8 ± 5.6*
Muscle	124 ± 44	209 ± 83*	81.4 ± 17.8	180 ± 92*
Lung	188 ± 46	278 ± 68*	4.6 ± 1.5	8.8 ± 2.8*
Heart	68.8 ± 16.2	86.6 ± 21.5	0.9 ± 0.3	1.8 ± 0.5*
Small intestine	401 ± 52	394 ± 65	52.4 ± 8.3	60.7 ± 11.1
Large intestine	252 ± 80	491 ± 119*	9.03 ± 3.0	19.3 ± 6.4*
Blood	40.1 ± 13.8	41.7 ± 0.1	10.2 ± 3.6	5.4 ± 1.8*
Total[3]			251	846

* Significantly different from pair-fed rats, $P < 0.05$.

[1] Two groups of rats were infected by an intravenous injection of live *Escherichia coli* ($6.5 \cdot 10^8$). One pair-fed control group was injected intravenously with saline and ate the same amount of food as the infected rats. Measurements were made 2 d after infection. Mean values ± SD are given for eight animals in pair-fed and infected rats Group 2 (except for values obtained in liver where $n = 5$ for infected rats Group 1).

[2] For absolute synthesis rate calculation in whole skeletal muscle, skeletal muscle was estimated to be 45% of body weight for the pair-fed rats (Miller 1969) and as 40% of body weight for the infected rats (Malmezat et al. 1998). For absolute synthesis rate calculation in whole blood, blood was estimated as 5.5% of body weight in the two groups (Miller 1969).

[3] The total amount of glutathione synthetized in the whole body was estimated by summing the total glutathione synthesis of the tissues examined and extrapolating the value to the body weight of the rat.

glutathione in the tissues. The infectious and inflammatory state, as well as nutritional status, modulate glutathione tissue levels (Cho et al. 1984, Hunter and Grimble 1997, Jahoor et al. 1995). However, the mechanisms that lead to these metabolic modifications are still poorly understood. The major aim of the present study was therefore to determine if changes in tissue glutathione concentrations observed during the early phase of sepsis in rats (2 d) could be partly explained by changes in glutathione ks.

Numerous studies have shown that tissue and plasma glutathione concentrations are depleted during either protein deficiency or food restriction (Cho et al. 1981 and 1984, Hum et al. 1992, Hunter and Grimble 1997, Jahoor et al. 1995). Such a decrease was also observed in this experiment in liver, since the level determined previously in well-fed rats was 5.18 μmol/g (Breuillé et al. 1994a). The decrease in glutathione concentration probably results from the decreased availability of substrates needed for glutathione synthesis, and particularly cysteine which is considered to be the most limiting amino acid (Deneke et al. 1983, Grimble et al. 1992). It has been demonstrated that protein-deficient diets produce a decrease in plasma, erythrocytes and liver glutathione ks (Hum et al. 1992, Hunter and Grimble 1997, Jahoor et al. 1995).

In spite of strong anorexia, glutathione concentration of septic rats was greater than pair-fed rats in nearly all tissues. Only the blood exhibited a decrease in glutathione content. Liver glutathione concentrations were even greater than the values we had measured in healthy fed rats (Breuillé et al. 1994a). Thus the inflammatory challenge overcame the influence of food restriction. Others have described unchanged or increased glutathione status in different tissues 24 and 48 h after induction of stress in animal models (Hunter and Grimble 1997, Jahoor et al. 1995). These results suggest that the organism is able to improve glutathione status through an increase in glutathione concentration in response to infection, that may represent a mechanism to protect against oxidative damage. Indeed, a higher rate of mortality has been observed in rats exposed to hyperoxia and glutathione depletion (Deneke et al. 1985).

Changes in the activities of glutathione reductase, peroxidase and transferase may have a bearing on the concentration of glutathione within tissues. A higher activity of glutathione reductase was observed in liver of septic compared to pair-fed rats. This agrees with the results of Hunter and Grimble (1997) who also showed that rats treated with tumor necrosis factor (TNF) exhibited an increase in the glutathione reductase activity. This response of liver to an inflammatory challenge may allow for the maintenance of a high GSH/GSSG ratio providing a high antioxidative potential. Peroxidase activities were little affected by infection. Thus, changes in the activity of reductase can have a greater potential impact on recycling of glutathione than alterations in peroxidase activity. The increased total liver GSH transferase activity in infected rats suggests an accelerated loss of glutathione from the body by the excretion of reduced glutathione conjugates, to protect cells against toxic compounds. However, as glutathione levels were greater in infected animals compared to pair-fed rats, rate of glutathione synthesis was probably more than adequate to support a potential higher rate of utilization. In the present study, the activity of γ-glutamyl-cysteine synthetase (limiting enzyme in glutathione synthesis) in whole liver was 50% higher in infected rats than in pair-fed controls. The difference in all these enzyme levels were small even when significant. However, there was no decrease of these enzymes, as is observed for negative acute phase proteins (Schreiber et al. 1982). Furthermore, measurements of enzyme activities indicate the capacity of the tissue for synthesis and does not consider the flow of available substrates through the enzymatic reaction and the presence of other modulating substances in vivo.

Few studies have been performed in vivo on glutathione synthesis in animal tissues. The methods used are often indirect (Hunter and Grimble 1997, Potter and Tran 1993). Only Jahoor et al. (1995 and 1999) have measured glutathione fractional ks using ^{2}H and ^{13}C glycine infusion. We have developed a selective and rapid procedure to determine enrichment of glutathione as a whole, and used labeled cysteine infusion to measure the glutathione fractional ks in tissues. However, the values are most likely lower than the actual rates, because of the uncertainties of the infusion method. The purpose of a constant infusion is to produce a constant enrichment of the precursor. As the calculation of ks requires the determination of the area under the curve describing the time course of the precursor enrichment, it can be very simply calculated if this constancy is achieved immediately. For cysteine, this was not the case. However, the area under the curve can be calculated more precisely, taking into account the time course of the rise of enrichment to plateau. The rate at which the plateau is reached depends upon the pool size of the labeled amino acid and is characterized by an empirically fitted rate constant. For lysine and tyrosine, this constant is 15 and 80 d^{-1}, respectively (Waterlow et al. 1978). As plasma cysteine concentration is between plasma lysine and tyrosine concentration, the value for cysteine that we have estimated to be 30 d^{-1} seems to be reasonable.

In liver of infected rats infused for 6 h, glutathione enrichment was very similar to free cysteine enrichment. There are several potential reasons for this. The enrichment of the immediate precursor pool for glutathione synthesis is different from that of the general free cysteine pool. Another possibility can be that glutathione ks is very fast in these rats, allowing glutathione enrichment to reach a plateau value at the same level as free cysteine within 6 h of infusion. In these conditions, the values obtained for glutathione ks underestimated the real values because we used the intracellular free cysteine as precursor for glutathione synthesis in the calculations. Therefore, we calculated liver glutathione ks in rats infused for 4 h. At this time, glutathione-bound cysteine enrichment has not reached a plateau, and this allowed us to obtain a better estimate. However, with both 4 and 6 h infusion, infection increased cysteine utilization for liver glutathione synthesis. Finally, the calculations do not take into account glutathione excretion and cysteine recycling from glutathione catabolism which are probably both important. Regardless, our data in liver are consistent with values previously reported. Using ^{13}C glycine infusion, Jahoor et al. (1995) reported a fractional ks of 175%/d in piglet liver 2 d after injection of turpentine. The apparent glutathione turnover in liver, determined from the decrease of glutathione-specific radioactivity after injection of ^{35}S cysteine, is about 350%/day in young rats (Potter and Tran 1993). By inhibiting glutathione synthesis using buthionine sulfoximine and assuming that the rate of glutathione disappearance reflects glutathione synthesis, Hunter and Grimble (1997) indirectly estimated glutathione synthesis in rat liver to be 440%/day.

Glutathione fractional ks were similar in the small intestine and blood of infected and pair-fed rats. Thus, there is a positive correlation in these tissues between fractional ks and glutathione concentrations, both not altered by infection. Similar results were observed in the intestine in pigs fed a protein-deficient diet (Jahoor et al. 1995). In other tissues, especially liver, glutathione ks was dramatically increased after infection.

This is in keeping with the increased glutathione concentration observed in this tissue, and is in accordance with the results of Hunter and Grimble (1997) in lipopolysaccharide injected rats. These authors have also shown that lipopolysaccharide treatment prevented the fall in liver glutathione level and synthesis due to the reduced food intake associated with the treatment. In our study, we have not included a well-fed group of rats and we cannot conclude if glutathione synthesis was restored to normal levels or enhanced above them. However, because glutathione levels in infected rats were close to those found in rats eating ad libitum (Breuillé et al. 1994a), we can assume that, despite lower food intake, the main effect of infection was to maintain glutathione concentration and synthesis.

The total amount of glutathione synthesized in the whole body can be estimated by summing the total glutathione synthesis of the tissues examined and extrapolating the value to the body weight of the rat. Calculated in this way, infection stimulated total glutathione synthesis 3.4-fold. From data previously obtained in our laboratory, we can estimate that 2.20 mmol of cysteine was incorporated daily into whole protein of septic rats vs. 1.39 mmol in pair-fed rats (Breuillé et al. 1994b and 1996). Thus, using these values, glutathione synthesis represents 38 and 18% of the amount of cysteine used for protein synthesis in infected and pair-fed rats, respectively. Therefore, glutathione synthesis accounts for 42% of the increased cysteine utilization 2 d after infection. Furthermore, it appears that about 64% of the plasma cysteine flux in septic rats is sustained by the turnover of glutathione compared to only 28% in pair-fed rats. In healthy men, glutathione synthesis may account for >50% of cysteine flux (Fukagawa et al. 1996).

Grimble et al. (1992) has suggested that sulfur amino acids play a key role in the economy of the body under inflammatory conditions. Following fractures and burns, urinary nitrogen excretion is enhanced to a greater extent than is sulfur excretion (Cuthbertson 1931, Larsson et al. 1982). Cysteine catabolism is decreased in numerous tissues following infection (Breuillé et al. 1996, Malmezat et al. 1998), suggesting a preferential retention of this amino acid in stress. Thus, taken together, these results suggest that cysteine requirements are increased during inflammatory states. Moreover, the present study shows that increased glutathione synthesis may be the primary cause of the increased cysteine requirement.

Glutathione homeostasis has been studied in numerous human diseases. In HIV infection, concentration of erythrocyte glutathione is decreased. This is due in part to a reduced ks secondary to a shortage in cysteine availability (Jahoor et al. 1999). As shown in our study and by Hunter and Grimble (1997), in the first days following an inflammatory challenge, tissue glutathione amounts were elevated in treated animals compared to control animals. However later on (data not shown) we observed depletion of tissue glutathione levels. Further studies on glutathione metabolism are therefore required to determine mechanisms responsible for glutathione deficiency in the late phase of infection and the effect of cysteine supplementation.

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LITERATURE CITED

Beutler, E. (1989) Nutritional and metabolic aspects of glutathione. Ann. Rev. Nutr. 9: 287–302.

Breuillé, D., Arnal, M., Rambourdin, F., Rosé, F. & Obled, C. (1996) Increased cysteine requirements induced by sepsis. Clin. Nutr. 15: 21.

Breuillé, D., Malmezat, T.,. Rosé, F., Pouyet, C. & Obled, C. (1994a) Assessment of tissue glutathione status during experimental sepsis. Clin. Nutr. 13: 5.

Breuillé, D., Rosé, F., Arnal, M., Melin, C. & Obled, C. (1994b) Sepsis modifies contribution of different organs to whole-body protein synthesis in rats. Clin. Sci. 86: 663–669.

Carlberg, I. & Mannervik, B. (1975) Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J. Biol. Chem. 250: 5475–5480.

Cho, E. S., Johnson, N. & Snider, B. C. (1984) Tissue glutathione as a cyst(e)ine reservoir during cystine depletion in growing rats. J. Nutr. 114: 1853–1862.

Cho, E. S., Sahyoun, N. & Stegink, L.D. (1981) Tissue glutathione as a cyst(e)ine reservoir during fasting and refeeding of rats. J. Nutr. 111: 914–922.

Colomb, V., Petit, J., Matheix-Fortunet, H., Heecketsweiler, B., Kaeffer, N., Lerebours, E. & Colin, R. (1995) Influence of antibiotics and food intake on liver glutathione and cytochrome P-450 in septic rats. Br. J. Nutr. 73: 99–110.

Cuthbertson, D. P. (1931) The distribution of nitrogen and sulfur in the urine during conditions of increased catabolism. Biochem. J. 25: 236–244.

Deneke, S. M. & Fanburg, B. L. (1989) Regulation of cellular glutathione. Am. J. Physiol. 257: L163–L173.

Deneke, S. M., Gershoff, S. N. & Fanburg, B. L. (1983) Potentiation of oxygen toxicity in rats by dietary protein or amino acid deficiency. J. Appl. Physiol. 54: 147–151.

Deneke, S. M., Lynch, B. A. & Fanburg, B. L. (1985) Effects of low protein diets or feed restriction on rat lung glutathione and oxygen toxicity. J. Nutr. 115: 726–732.

Dröge, W., Pottmeyer-Gerber, C., Schmidt, H. & Nick, S. (1986) Glutathione augments the activation of cytotoxic T lymphocytes in vivo. Immunobiology 172: 151–156.

Fagan, J. M., Ganguly, M., Tiao, G., Fischer, J. E. & Hasselgren, P. O. (1996) Sepsis increases oxidatively damaged proteins in skeletal muscle. Arch. Surg. 131: 1326–1331.

Fukagawa, N. K., Ajami, A. M. & Young, V. R. (1996) Plasma methionine and cysteine kinetics in response to an intravenous glutathione infusion in adult humans. Am. J. Physiol. 270: E209–E214.

Gérard-Monnier, D. & Chaudière, J. (1996) Metabolism and antioxidant function of glutathione. Pathol. Biol. 44: 77–85.

Grimble, R. F., Jackson,A. A., Persaud, C., Wride, M. J., Delers, F. & Engler, R. (1992) Cysteine and glycine supplementation modulate the metabolic response to tumor necrosis factor alpha in rats fed a low protein diet. J. Nutr. 122: 2066–2073.

Habig, W. H., Pabst, M. J. & Jakoby, W. B. (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249: 7130–7139.

Hum, S., Koski, K. G. & Hoffer, L. J. (1992) Varied protein intake alters glutathione metabolism in rats. J. Nutr. 122: 2010–2018.

Hunter, E.A.L. & Grimble, R. F. (1997) Dietary sulphur amino acid adequacy influences glutathione synthesis and glutathione dependent enzymes during the inflammatory response to endotoxin and tumour necrosis factor-alpha in rats. Clin. Sci. 92: 297–305.

Ikegami, K., Lalonde, C., Youn, Y. K., Picard, L. & Demling, R. (1994) Comparison of plasma reduced glutathione and oxidized glutathione with lung and liver tissue oxidant and antioxidant activity during acute inflammation. Shock 1: 307–314.

Jahoor, F., Jackson, A., Gazzard, B., Philips, G., Sharpstone, D., Frazer, M. E. & Heird, W. (1999) Erythrocyte glutathione deficiency in symptom-free HIV infection is associated with decreased synthesis rate. Am. J. Physiol. 276: E205–E211.

Jahoor, F., Wykes, L. J., Reeds, P. J., Henry, J. F., Del Rosario, M. P. & Frazer, M.E. (1995) Protein-deficient pigs cannot maintain reduced glutathione homeostasis when subjected to the stress of inflammation. J. Nutr. 125: 1462–1472.

Kan, B., London, I. M. & Levin, D. H. (1988) Role of reversing factor in the inhibition of protein synthesis initiation by oxidized glutathione. J. Biol. Chem. 263: 15652–15656.

Kataoka, H., Takagi, K. & Makita, M. (1995) Determination of glutathione and related aminothiols by gas chromatography with flame photometric detection. Biomed. Chromatogr. 9: 85–89.

Keller, G. A., Barke, R., Harty, J. T., Humphrey, E. & Simmons, R. L. (1985) Decreased hepatic glutathione levels in septic shock. Arch. Surg. 120: 941–945.

Larsson, J., Liljedahl, S., Martenson, J., Nordstrom, H., Schildt, B. &. Sarbo, B. (1982) Urinary excretion of sulfur amino acids and sulfur metabolites in burned patients receiving parenteral nutrition. J. Trauma 2: 656–663.

Luo, J. L., Hammarqvist, F., Andersson, K. & Wernerman, J. (1998) Surgical trauma decreases glutathione synthetic capacity in human skeletal muscle tissue. Am. J. Physiol. 275: E359–E365.

Malmezat, T., Breuillé, D., Pouyet, C., Patureau Mirand, P. & Obled, C. (1998) Metabolism of cysteine is modified during the acute phase of sepsis. J. Nutr. 128: 97–105.

Miller, S. A. (1969) Protein metabolism during growth and development. In: Mammalian Protein Metabolism (Munro. H. N., ed.) vol. 3, pp. 183–233. Academic Press: New York, NY.

Paglia, D. E. & Valentine, W. N. (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70: 158–169.

Peralta, J. G., Llesuy, S., Evelson, P., Carreras, M. C, Flecha, B. G & Poderoso, J. J. (1993) Oxidative stress in skeletal muscle during sepsis in rats. Circ. Shock 39: 153–159.
Potter, D. W. & Tran, T. B. (1993) Apparent rates of glutathione turnover in rat tissues. Toxicol. Appl. Pharmacol. 120: 186–192.
Rouzer, C. A., Scott, W. A., Hamill, A. L., Liu , F. T., Katz, D. H. & Cohn, Z. A. (1982) Secretion of leukotriene C and other arachidonic acid metabolites by macrophages challenged with immunoglobulin E immune complexes. J. Exp. Med. 156: 1077–1086.
Schreiber, G., Howlett, G., Nagashima, M., Millership, A., Martin, H., Urban, J. & Kotler, L. (1982) The acute phase response of plasma protein synthesis during experimental inflammation. J. Biol. Chem. 257: 10271–10277.
Seelig, G. F. & Meister, A. (1985) Glutathione biosynthesis; γ-glutamyl-cysteine synthetase from rat kidney. Meth. Enzymol. 113: 379–390.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Gartner, A. K., Provenzano, M. D., Fuijimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. Anal. Biochem. 150: 76–85.
Staal, F.J.T, Ela, S. W., Roederer, M., Anderson, M. T., Herzenberg, L. A. & Herzenberg, L. A. (1992) Glutathione deficiency and human immunodeficiencyvirus infection. Lancet 339: 909–912.
Suthanthiran, M., Anderson, M. E., Sharma ,V. K. & Meister, A. (1990) Glutathione regulates activation-dependent DNA synthesis in highly purified normal human T lymphocytes stimulated via the CD2 and CD3 antigen. Proc. Natl. Acad. Sci. USA 87: 3343–3347.
Waterlow, J. C., Garlick, P. J. & Millward, D. J. (1978) Protein turnover in mammalian tissues and in the whole body. pp. 804. North-Holland Publishing Company, Amsterdam.