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INFLUENCE OF CYSTEINE UPON THE GLUTATHIONE STATUS OF ISOLATED RAT HEPATOCYTES

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Abstract—Contrary to a previous report [J. Viña, R. Hems and H. A. Krebs, *Biochem. J.* **170**, 627 (1978)], we have observed that incubation of isolated rat hepatocytes in a medium containing high concentrations of cysteine did not deplete intracellular glutathione (GSH), but instead caused a net increase of GSH. Hepatocyte-dependent accumulation of the mixed disulfide of cysteine and GSH (CYSSG) in incubation media within a 2-hr period was increased several-fold in medium containing cysteine or cystine compared to methionine-containing medium. Hepatocytes (10^6 cells/ml) placed in medium containing normal levels of cysteine, cystine, or methionine established about a 1:1 ratio of extracellular GSH to GSSG at a concentration of about 6 μM each. These results indicate a possible intracellular–extracellular relationship for glutathione and an intracellular response to the extracellular status of glutathione and cyst(e)ine.

The mammalian liver has an important role in providing glutathione (GSH) for cellular protection against reactive intermediates formed during the metabolism of many foreign chemicals, as well as being a major site for glutathione conjugation reactions [1, 2]. Recent studies indicate that the liver may also have a major role in supplying plasma glutathione for interorgan glutathione relationships. Regulation and maintenance of both intracellular and extracellular levels of liver glutathione, therefore, have become increasingly important considerations which have been studied, in part, by the use of freshly isolated hepatocytes [3–5]. In this regard, high doses of cysteine, administered *in vivo* to rats, failed to increase liver GSH content while doubling the non-protein thiol content for a period of 1–2 hr [6]. In contrast, isolated hepatocytes have been reported to undergo extensive loss of GSH during incubation in medium containing 0.25 to 4.0 mM cysteine. This phenomenon was hypothesized to result from oxidation of the cysteine to cystine which reacted with intracellular GSH to yield CYSSG (the mixed disulfide of cysteine and GSH) [7].

We wish to report that isolated hepatocytes incubated in a complete medium containing 0.16 to 3.0 mM cysteine did not lose GSH but increased intracellular GSH while providing glutathione for extracellular CYSSG accumulation. In addition, under normal conditions hepatocytes appear to establish quickly and maintain an intracellular to extracellular GSH ratio of about 1000 to 1.

MATERIALS AND METHODS

Hepatocytes were isolated from fed, male Sprague–Dawley rats, 180–250 g body wt, as described previously [5] with the following modifications. Albumin was omitted from the collagenase perfusion and from the EGTA† perfusion buffers in experiments with high levels of cysteine. Collagenase (Type IV, Sigma Chemical Co., St. Louis, MO) perfusion time was 10–12 min. Aliquots of freshly prepared hepatocytes were counted on a hemacytometer using 0.14% trypan blue (Harleco, Santa Ana, CA) containing 2% bovine serum albumin (Sigma Chemical Co.) and were diluted to 1–2 × 10^6 cells/ml in Fischer's medium lacking cystine and methionine [8], to which sterile, heat-inactivated bovine (Gibco, Grand Island, NY) fetal calf serum (FCS) was added to a final concentration of 10%. Cells were incubated at 37° in a gyratory shaker under an atmosphere of 5% CO_2–95% O_2 in 25 ml Erlenmeyer flasks capped with serum stoppers. Sulfur-containing amino acids were added to the incubation medium at the concentrations indicated in the appropriate table. The addition of 10% FCS did not result in the presence of detectable (<1 nmole/ml) free sulfur amino acids.

Lactate dehydrogenase (LDH) leakage (for determination of cell viability) was assayed hourly with a Beckman TR Analyzer [9]. It was found necessary to sonicate (3 sec) cells to which Triton X-100 had been added to effect total release of intracellular LDH. Hepatocyte preparations with 5–7% LDH leakage were used for experiments.

Samples of cells and medium were derivatized to form the dinitrophenylated *S*-carboxymethyl (of thiol-containing compounds only) products for h.p.l.c. as described previously [10]. Hepatocytes (1–2 × 10^6 cells/ml) were removed from the incubation mixtures by centrifugation (50 *g* for 4 min), and the cell pellet was washed once in 0.9% saline. The cell pellet was resuspended in 1 ml of 0.9% saline, 0.05 ml of 70% perchloric acid was added, and the protein was removed by centrifugation. A 0.5 ml aliquot of the supernatant fraction was treated immediately with 4 μmoles of iodoacetic acid and then was neutralized with an excess of $NaHCO_3$. After 15 min at room temperature, 0.5 ml of an alcoholic solution of 1-fluoro-2,4-dinitrobenzene [1.5:98.5 (v/v) of 1-fluoro-2,4-dinitrobenzene–abso-

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† EGTA = ethyleneglycolbis (amino-ethylether)tetra-acetate.

lute ethanol] was added, and the reaction was allowed to proceed for 4 hr in the dark. A 1-ml sample of the incubation medium was deproteinized with 0.05 ml of 70% perchloric acid, and a 0.5 ml aliquot of the acid supernatant fraction was treated similarly to that from cells.

Constituents of cells and medium were analyzed after derivatization by injection of an aliquot onto a 4 × 250 mm amine-based anion-exchange column, either Micro Bondapak amine (Waters Associates, Milford, MA) or Spherisorb S5W (Phase Separations Ltd., U.K.), derivatized with 3-amino-propyltriethoxysilane and packed in our laboratory. The 2,4-dinitrophenyl (DNP) derivatives were separated by gradient elution (0.05 to 0.4 M sodium acetate, pH 4.6, in 80% methanol). The flow rate was 2 ml/min, and the gradient volume was 60–90 ml depending upon the age of the column. The h.p.l.c. system consisted of a Spectra-Physics model 3500 liquid chromatograph equipped with a u.v. detector (350 nm) and a Spectra-Physics System I integrator. All derivatives were quantitated by internal standardization with authentic standards.

Bovine erythrocyte superoxide dismutase and bovine liver catalase were purchased from the Sigma Chemical Co. Data are expressed as means ± S.D. (one sigma error) obtained from three different experiments conducted in duplicate.

RESULTS

Intracellular GSH content approximately doubled within 2 hr when isolated hepatocytes were incubated in a complete medium containing cysteine (0.5 to 3.0 mM) and were compared with hepatocytes incubated in a medium devoid of sulfur amino acids (Table 1). The maximum increase in intracellular GSH content (22.5 nmoles/10^6 cells per 2 hr) occurred when both methionine (0.67 mM) and cysteine (3.0 mM) were present in the medium (Table 1). No evidence was obtained for GSH depletion during incubations in which almost complete oxidation of cysteine to cystine occurred in 2 hr (Table 2). The fraction of cysteine oxidized in a 2-hr period was essentially the same when the cysteine concentration was varied from 0.5 to 3.0 mM. Analytical difficulties, possibly due to limited cystine solubility, contributed to the lack of recovery of cystine at 120 min (Table 2). Cysteine uptake doubled the total intracellular cysteine plus GSH content of the hepatocytes within 20 min, after which a decrease of intracellular cysteine to about thrice normal levels by 2 hr occurred (Table 2).

Table 1. Intracellular GSH content of hepatocytes incubated in formulated Fischer's medium with added sulfur amino acids

Addition of L-cysteine (mM)	Methionine (mM)	GSH* (nmoles/10^6 cells) 60	120
0	0	23.1 ± 3.8	20.9 ± 2.7
0	0.67	37.6 ± 5.3	43.6 ± 4.7
0.5	0	33.1 ± 1.7	38.3 ± 6.9
1.0	0	37.5 ± 5.4	36.2 ± 1.1
2.0	0	33.7 ± 3.9	37.2 ± 7.3
3.0	0	35.1 ± 4.5	39.5 ± 2.6
3.0	0.67	44.9 ± 15.3	52.3 ± 5.9

* Initial intracellular GSH content was 29.8 ± 3.1 nmoles/10^6 cells. Data are X ± S.E.M. for N = 6.

Table 2. Fate of cysteine during incubation of isolated hepatocytes*

Time (min)	Cystine Extracellular (nmoles/ml)	Cysteine Extracellular (nmoles/ml)	Cysteine Intracellular†‡ (nmoles/10^6 cells)
10	134 ± 30	2862 ± 241	35.7 ± 21.8
20	376 ± 58	2585 ± 148	50.2 ± 12.4
40	686 ± 62	1794 ± 490	32.2 ± 3.4
60	841 ± 73	1423 ± 247	19.9 ± 4.7
120	1099 ± 200	310 ± 54	3.4 ± 1.5

* Fischer's medium containing 3.0 mM cysteine and 0.67 mM methionine as the sulfur amino acids.

† Intracellular cysteine content was normally less than 1 nmole/10^6 cells.

‡ Intracellular cystine was not quantitated in these experiments due to the lack of resolution when relating a small quantity of cystine to a much larger glutamate content.

Extracellular accumulation of CYSSG occurred during incubation of hepatocytes in media containing sulfur amino acids. Although the increase was significant with methionine in the medium (6 nmoles/10^6 cells per 2 hr), a much greater increase of CYSSG was observed with either cysteine or cystine in the medium (17–24 nmoles/10^6 cells per 2 hr) (Table 3). CYSSG accumulation was essentially linear with time up to 2 hr even when cystine was 0.08 mM or cysteine was 0.16 to 3.0 mM. Total glutathione change per 10^6 cells in 2 hr, as indicated (Table 3), ranged from a decrease of 2 nmoles to an increase of 43 nmoles in 2 hr (Table 3).

Hepatocytes were incubated, in formulated Fischer's medium that lacked methionine but contained 3 mM cysteine, in the presence of various agents to examine their possible effects upon CYSSG formation. These agents—ascorbate, 1 mg/ml; catalase, 50,000 units/ml; or superoxide dismutase, 900 units/ml—were all without effect on the accumulation of CYSSG in the medium over a 2-hr period.

Medium containing basal levels of cysteine (0.16 mM), cystine (0.08 mM), or methionine (0.67 mM) was analyzed at 10, 30, 60 and 120 min after addition of isolated hepatocytes. The rapid appearance of GSH in the media within 10 min was followed by a much slower increase, reaching 6–7 nmoles/10^6 cells in 120 min (Table 4). The GSSG content of the medium was much lower (1 nmole/10^6 cells) at 10 min but increased to 5–6 nmoles/10^6 cells within 2 hr). A high cysteine content (3.0 mM) in the medium caused the levels of GSH and GSSG to remain below detectable levels (Table 4).

Thiol-disulfide interchange reactions during non-enzymic O_2 oxidation of cysteine and GSH were examined in media without hepatocytes. Incubations of GSH (0.2 mM), or GSH (0.2 mM) and cysteine

Table 3. Accumulation of extracellular mixed disulfide of cysteine and glutathione (CYSSG) during incubation of hepatocytes in formulated Fischer's medium containing added sulfur amino acids

Amino acid addition	Concn (mM)	CYSSG* (nmoles/ml per 2 hr)	Total glutathione change (nmoles/10^6 cells per 2 hr)
None		0.9 ± 0.3	−2.4†
Cystine	0.08	22.3 ± 1.7	5.1‡
Methionine	0.67	6.4 ± 1.3	12.6‡
Cysteine	0.16	19.1 ± 2.0	27.8‡
Cysteine	0.5	17.4 ± 1.4	24.8†
Cysteine	1.0	19.8 ± 1.6	25.1†
Cysteine	2.0	16.6 ± 1.9	22.9†
Cysteine	3.0	23.9 ± 8.0	39.2†
Cysteine and methionine	3.0 0.67	21.3 ± 10.1	42.7†

* Initial extracellular CYSSG was 1.1 ± 1.1 nmoles/ml when 10^6 cells/ml were present. Intracellular CYSSG was less than 1 nmole/10^6 cells at 2 hr.

† Total increase of GSH equivalents was measured as extracellular and intracellular GSH plus CYSSG.

‡ Total increase of GSH equivalents was measured as extracellular and intracellular GSH and GSSG plus CYSSG.

Table 4. Extracellular GSH and GSSG content of medium during incubation of hepatocytes in formulated Fischer's medium containing the indicated sulfur amino acids

Amino acid addition	Concn (mM)	GSH (nmoles/ml)* Time (min) 10	GSH 120	GSSG 10	GSSG 120
Cysteine	0.16	3.1 ± 1.0	6.7 ± 1.3	< 0.3	5.5 ± 2.6
Cysteine	3.0	< 0.3	< 0.3	< 0.3	< 0.3
Cystine	0.08	5.4 ± 0.5	6.5 ± 1.8	1.0 ± 0.3	5.6 ± 3.1
Methionine	0.67	3.1 ± 0.1	6.6 ± 2.4	< 0.3	4.7 ± 1.6

* The data are per ml of medium containing 10^6 cells/ml; X ± S.E.M., where N = 6.

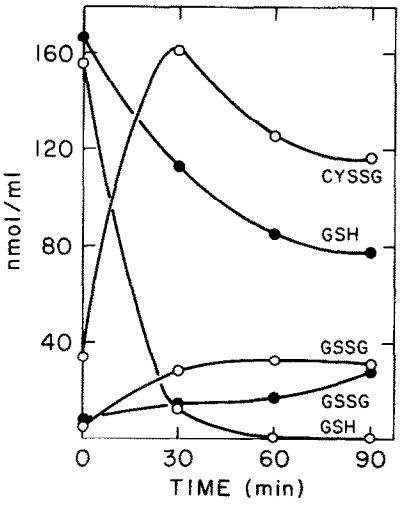

Fig. 1. Incubation of 0.2 mM glutathione (—●—), or glutathione (0.2 mM) and cysteine (0.16 mM) (—○—), in Fischer's medium devoid of methionine and cystine but containing 10% fetal calf serum in a 95% O_2–5% CO_2 atmosphere at 37°. Aliquots of the incubation mixture were analyzed by the h.p.l.c. procedure described under Materials and Methods.

(0.16 mM), in Fischer's medium devoid of cystine and methionine but containing 10% fetal calf serum, resulted in a several-fold greater rate of GSH disappearance when cysteine was present in the medium (Fig. 1). Without cysteine in the incubation medium, GSH was converted to GSSG. When cysteine was present, rapid cysteine oxidation was accompanied by essentially quantitative CYSSG formation and by an accelerated rate of GSH consumption when compared to the rate of GSH oxidation to GSSG (Fig. 1).

DISCUSSION

Assays of aliquots of hepatocytes and incubation media after incubation of hepatocytes in media containing cysteine concentrations as high as 3.0 mM demonstrated a net synthesis of intracellular GSH. These findings are in marked contrast to a previous report [7] in which hepatocytes underwent almost complete depletion of intracellular GSH when incubated in the presence of high cysteine concentrations. It would appear from that report, however, that the cells were not separated from the medium prior to assay for GSH [7]; the possibility of CYSSG formation was discussed, but as a phenomenon that

occurred during incubation rather than as a result of the sample preparation procedure. In addition, the analytical method employed did not measure the glutathione that may have been present as CYSSG [7].

Cultured human diploid fibroblasts export GSH into cystine-free medium until the total glutathione content of the medium reaches about 1–3 μM [11]. The presence of cystine (0.05 mM) in the medium resulted in CYSSG accumulation [11], confirming similar findings with hepatocytes incubated in medium containing normal concentrations of cystine [4]. At high concentrations (up to 3.0 mM), cysteine, which underwent oxidation to cystine (Table 2), failed to cause an accumulation of CYSSG in the medium greater than that caused by cystine at 0.08 mM in the medium. Moreover, rather than causing a decrease in intracellular GSH as shown with 0.08 mM cystine [4], high cysteine concentrations caused an influx of cysteine into the hepatocytes and a net synthesis of intracellular GSH (Tables 1 and 3). Assuming that the hepatocyte intracellular GSH content ranges from about 3 to 6 mM and that there is a concentration of about 6 μM in the various media (Table 4), an intracellular to extracellular GSH gradient of 300 to 1000 would be established during incubation of hepatocytes in the various media that were varied in their content of sulfur-containing amino acids. This calculation agrees quite well with human diploid fibroblasts which maintained an intracellular GSH content of 3 mM with an extracellular GSH level of 3 μM [11].

A thiol-disulfide interchange reaction between glutathione and cystine has been shown to occur as follows:

$$\text{Cystine} + \text{GSH} \rightleftharpoons \text{CYSSG} + \text{cysteine} \quad (1)$$

$$\text{CYSSG} + \text{GSH} \rightleftharpoons \text{GSSG} + \text{cysteine} \quad (2)$$

with the equilibrium constants being 3.2 and 1.2, respectively, at 37° and pH 7.4 [12]. In our experiments, the concentration of cystine in normal medium was 0.08 mM, thus assuring that more than 90 per cent of the glutathione present in the medium was present as CYSSG. At 3.0 mM cysteine all of the detectable glutathione was present as CYSSG.

Certain non-enzymic thiol-disulfide interchange reactions occur rapidly. Even at a low cysteine concentration (0.16 mM), the non-enzymic formation of CYSSG was extensive in 30 min as cysteine oxidation occurred in the presence of GSH (Fig. 1).

These reactions may be important in interorgan glutathione relationships, for there is an efflux of GSH from perfused liver [13, 14], and the kidney appears to play a major role in the metabolic turnover of extracellular glutathione and glutathione-mixed disulfides [15–18]. The glutathione concentration has been found to be 3–7 μM in rat plasma [14, 19, 20], due mainly to a renal extraction efficiency for glutathione of about 90 per cent, with a renal filtration efficiency of about 30 per cent [18]. The degradation of glutathione was postulated to occur mainly on the luminal surface of the renal brushborder membrane [21]. These findings are supported by glutathione degradation studies with rat isolated kidney cell preparations. Incubations of glutathione with kidney cells have shown that γ-glutamyl transpeptidase functions as a glutathionase and that cystinylglycine dipeptidase hydrolyzes the glycine dipeptide [22–24]. In this regard, CYSSG has been shown to be degraded as rapidly as GSH or GSSG by extracellular γ-glutamyl transpeptidase [24].

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