Glutathione is required for intestinal function

(mitochondria/buthionine sulfoximine/glutathione monoester/pancreas)

JOHANNES MÅRTENSSON*, AJEY JAIN†, AND ALTON MEISTER*

Departments of *Biochemistry and †Pediatrics, Cornell University Medical College, 1300 York Avenue, New York, NY 10021

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ABSTRACT Glutathione (GSH) deficiency produced in mice by giving buthionine sulfoximine leads to severe degeneration of the epithelial cells of the jejunum and colon. This is prevented by giving GSH monoester (orally or i.p.) and also by giving GSH (orally, but not i.p.). The i.p. administration leads to high plasma levels of GSH but does not appreciably increase GSH levels in intestinal mucosa or pancreas. These and previous studies on lens, lung, lymphocytes, liver, heart, and skeletal muscle indicate that there is very little, if any, transport of intact GSH from plasma to these tissues. Cells can use extracellular GSH by a pathway involving its cleavage, uptake of products and intracellular GSH synthesis. Epithelial cells of the gastrointestinal tract may use this pathway and can also take up luminal GSH (which arises partly from the bile) by a mechanism(s) that may involve transport of dipeptides or of GSH. It is suggested that biliary GSH normally functions in the protection of intestinal mucosa. Administration of GSH may be protective of the gastrointestinal epithelium and may also serve as a good source of cysteine moieties for intracellular GSH synthesis in the gastrointestinal tract and in other tissues. Administration of GSH delivery agents such as GSH esters is more effective than administration of GSH in increasing cellular and mitochondrial levels of GSH.

Administration to experimental animals of L-buthionine-SR-sulfoximine (BSO), a transition-state inhibitor of γ -glutamylcysteine synthetase, leads to decreased synthesis of glutathione (GSH) in many tissues (1–4). Decreased cellular GSH levels and capacity for GSH synthesis sensitize cells to radiation and to certain drugs; these effects provide a potentially useful approach in cancer therapy (5–12). Administration of BSO offers several advantages over other methods of cellular GSH depletion, one of which is that it makes possible the selective decrease of cellular GSH for relatively long periods, thus facilitating study of the effects of GSH deficiency on cell structure and function. Administration of BSO to mice for 9–21 days, without application of stress, led to marked structural changes in skeletal muscle (13), lung (14), and epithelial cells of newborn lens (15); such effects were not found in heart (13) and liver (14). Mitochondrial damage is found in tissues affected by GSH deficiency (13–15); the mitochondrial levels of GSH in tissues markedly affected by GSH depletion (lung, muscle, lens) were $\approx 20\%$ of the untreated controls, whereas in heart and liver the mitochondrial GSH levels were $>40\%$ of the controls. Mitochondrial and other cellular damage was prevented by simultaneous administration of GSH monoester but not by administration of GSH. GSH monoesters can enter cells effectively and are converted to GSH intracellularly (16–19). In contrast, GSH is poorly transported into cells.

These studies were stimulated by the observation (13) that administration of BSO to 28- to 30-g mice for 9–21 days led to a marked decrease in weight gain and that simultaneous

administration of BSO and GSH monoester gave weight gains that were $\approx 85\%$ of those found in controls; GSH had less effect on weight gain. The mice given BSO were found to have diarrhea and markedly decreased levels of GSH in the gastrointestinal tract (14). As described here, these animals showed severe degeneration of intestinal mucosal epithelial cells.

EXPERIMENTAL PROCEDURES

Materials. Mice (male; 34–38 g, Swiss-Webster from Taconic Farms) were maintained on Purina Chow. BSO (1, 20, 21), L-buthionine sulfone (21), and GSH monoethyl ester- $\frac{1}{2}\text{H}_2\text{SO}_4$ (16, 19, 22) were prepared as described. Aqueous solutions of GSH monoethyl ester were adjusted to pH 6.5–6.8 by cautious addition of 2 M NaOH just prior to injection; controls were given solutions containing equimolar levels of GSH, ethanol, and Na_2SO_4 .

Methods. Mice were sacrificed by i.p. administration of xylazine (5 mg/kg) and ketamine (50 mg/kg). Within 3 min, the viscera were perfused from the left ventricle (after opening the right ventricle) with 10 ml of cold saline. The organs became pale after 1–2 min, and the stomach (mid portion), jejunum, ascending colon, and pancreas were excised and profusely rinsed with 10–20 ml of cold saline. The gastric and intestinal mucosa were obtained by gentle scraping with a spatula. The tissues were homogenized in 5 vol of 5% 5-sulfosalicylic acid. The homogenates were centrifuged for 3 min at $10,000 \times g$ at 4°C and the supernatant solutions were immediately analyzed for total GSH by the GSH disulfide reductase 5,5'-dithiobis(2-nitrobenzoic acid) recycling procedure (23, 24).

The mitochondrial fraction of jejunal mucosa was isolated (from samples obtained by pooling mucosa from three mice) (13–15, 25–27) and checked by electron microscopy. Electron microscopy (13, 28), determinations of citrate synthase activity (25, 26), and protein (29) were carried out as described.

RESULTS

Effect of BSO on GSH Levels. Administration of BSO led to a rapid decline of the GSH levels of each of the tissues examined (Fig. 1). The estimated $t_{1/2}$ values for the initial rates of decline (cf. refs. 13 and 14) are 35, 40, 40, and 30 min, respectively, for jejunal mucosa, colon mucosa, gastric mucosa, and pancreas; these values, which are similar to those found for lymphocytes (14), lung (14), liver (3, 14), and kidney (3), reflect a relatively rapid rate of GSH synthesis *in vivo*. As also noted for other tissues, the initial rapid decrease in GSH level is followed by a much slower rate of decrease, indicating that there are fractions of cellular GSH that turn over slowly and that may be sequestered within cell organelles such as mitochondria (30). Depletion of GSH occurred at a slower

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Abbreviations: GSH, glutathione; BSO, L-buthionine-SR-sulfoximine; p.o., orally.

FIG. 1. Effect of inhibition of GSH synthesis on GSH levels. Mice were given BSO (2.5 mmol/kg i.p. and 2.5 mmol/kg by gastric lavage) twice daily (at 9 a.m. and 4 p.m.) for 14 days. BSO was added to the drinking water (10 mM). The controls were treated in the same way with an equivalent volume of saline and were given tap water to drink. The initial values (100%; zero time) were (means $\pm$ SD; $n = 3-5$) 2.92 ± 0.35 (jejunal mucosa), 1.73 ± 0.29 (gastric mucosa), 1.70 ± 0.13 colon mucosa, and 1.71 ± 0.18 (pancreas) $\mu\text{mol/g}$. The values for the controls were within 10% of those of the corresponding initial values; the first time point is 2 hr. (Inset) Levels found after a single administration of BSO (p.o. and i.p., as described above). Curves: 1, gastric mucosa; 2, jejunal mucosa; 3, pancreas; 4, colon mucosa.

rate in gastric mucosa than in the other tissues examined, and the rate of increase of the GSH levels after discontinuation of BSO treatment was greatest in the gastric mucosa.

Epithelial Cell Damage Associated with GSH Deficiency. After treatment of mice with BSO (as in Fig. 1) for 7 days, electron microscopy showed substantial ($\approx 50\%$) loss of height of the epithelial cell layer in the jejunal mucosa and colon mucosa (Fig. 2). There was marked mucosal damage with microvillus desquamation, mitochondrial swelling, mitochondrial degeneration, and vacuolization. None of these changes was seen in comparable studies in which mice were given equimolar amounts of buthionine sulfone in place of BSO. The changes found after giving BSO for 14 days are apparently reversible; they were not seen in mice that were treated with BSO for 14 days and then allowed to recover for 6 weeks. Additional evidence consistent with mitochondrial damage was found in studies on citrate synthase in jejunal mitochondria. Citrate synthase activity decreased from $1.15 \pm 0.13 \mu\text{mol per min per mg of protein}$ in untreated mice to $0.45 \pm 0.03 \mu\text{mol per min per mg of protein}$ in jejunal mitochondria from mice treated with BSO for 7 days.

No consistent morphological changes were observed in the pancreas or stomach by electron microscopy after 7 days of treatment with BSO.

Effect of Treatment with GSH Ester or with GSH. After treatment with both BSO and GSH ester for 7 days, there was virtually complete absence of the morphological changes found after giving BSO alone. Such protection was found when GSH ester was given i.p. and also when it was given orally (p.o.). [GSH monoester is taken up intact after oral administration (18).] In contrast, treatment with BSO plus GSH (i.p.) led to findings that were essentially the same as those after giving BSO alone. Treatment with BSO plus GSH

(p.o.) led to findings similar to those observed after treatment with BSO and GSH ester—i.e., almost complete absence of morphological damage. When BSO plus cysteine, glycine, and glutamate were given, there was no protection.

Studies on citrate synthase activities of jejunal mitochondria showed that this activity, which was $0.45 \pm 0.03 \mu\text{mol per min per mg of protein}$ after treatment with BSO, was $0.92 \pm 0.16 \mu\text{mol per min per mg of protein}$ after treatment with BSO plus GSH ester (p.o.) and $0.64 \pm 0.10 \mu\text{mol per min per mg of protein}$ after treatment with BSO plus GSH (p.o.).

The GSH levels of several tissues and of isolated jejunal mitochondria were determined after 7 days of treatment with BSO, BSO plus GSH monoester, BSO plus GSH, and BSO plus a mixture containing glycine, glutamate, and cysteine (Table 1). The GSH levels of the jejunal, colon, and gastric mucosa and of the pancreas were substantially higher when BSO plus GSH ester was given than when BSO was given alone (controls). GSH ester was effective when given i.p. and also when given by gastric lavage (p.o.). In the studies on pancreas, administration of BSO plus GSH (p.o. or i.p.) or a mixture (p.o.) of glutamate, glycine, and cysteine led to slightly higher GSH levels than found after giving only BSO. In the studies on jejunum and colon, administration of BSO plus GSH (i.p.) did not lead to significantly higher levels of GSH as compared to those obtained after giving BSO alone; however, the GSH levels found after giving BSO plus GSH (p.o.) were significantly greater than found after giving BSO alone. The values for gastric mucosa indicate that giving BSO plus GSH (p.o. or i.p.) leads to significantly higher GSH levels than found after giving BSO alone. The level of GSH in jejunal mucosal mitochondria was higher after giving BSO plus GSH ester (p.o.) than after giving BSO plus GSH (p.o.).

In summary, administration of GSH monoester increased GSH levels of all of the tissues whether given p.o. or i.p. Administration of GSH i.p. increased gastric mucosa GSH to some extent but otherwise did not appreciably affect the tissue levels of GSH despite the fact that the plasma levels of GSH were very greatly increased. Oral administration of GSH increased GSH levels in jejunum, colon, and stomach but less so than administration of GSH ester. Mechanisms by which GSH administered p.o. might increase the GSH levels of the mucosal cells are considered below.

It is notable that administration of GSH ester by gastric lavage increased the level of jejunal mitochondrial GSH to a value slightly higher than that of untreated mice, whereas the total GSH level of the jejunal mucosa, though increased, was less than that of untreated mice. Similar observations were made in studies on mitochondria isolated from heart (13), skeletal muscle (13), liver (14), and lung (14). These findings suggest that there is an active uptake of GSH by mitochondria from the cytosol (cf. ref. 30).

Effects on Weight Gain. Untreated mice gained weight at an average of 3.3 g per 10 days. Mice treated with BSO gained 0.4 g per 10 days. The weight gains were 1.0 g per 10 days for mice given BSO plus GSH (i.p.) and 2.4 g per 10 days for mice given BSO plus GSH (p.o.). Mice given BSO plus GSH ester gained 2.5 g per 10 days (i.p.) and 2.6 g per 10 days (p.o.). Thus, oral administration of GSH or of its monoester restored $\approx 78\%$ of the weight loss associated with giving BSO alone. Previously, it was found that GSH administered i.p. had much less effect on the weight gain of BSO-treated mice than did administration of GSH ester (13).

Related Studies. GSH administered i.p. to untreated mice does not greatly affect within 1–3 hr the GSH levels of liver (14, 16), kidney (16), lymphocytes (14), and lung (14). In the present study, we found that administration to mice (not treated with BSO) of GSH (4 mmol/kg) by gastric lavage did not appreciably affect the GSH levels of jejunal mucosa, colon mucosa, and pancreas—i.e., no more than $\approx 20\%$ increase in 2 hr; the GSH level of the gastric mucosa was

FIG. 2. Representative electron micrographs of the jejunal (*a-c*) and colon (*d-f*) mucosa in mice treated with BSO (*a* and *d*), BSO plus GSH monoethyl ester (*b* and *e*), and BSO plus GSH (*c* and *f*). Other micrographs showed that degeneration was greater in villus tip cells, which have a lower level of GSH (and a higher level of γ -glutamyltranspeptidase), than in the villus crypt cells (31). In *a* and *d*, there is a marked loss of microvilli, epithelial height, and mitochondria with vacuolization, mitochondrial degeneration, and nuclear changes. In *b*, *c*, *e*, and *f*, the epithelia resemble normal jejunal (*b* and *c*) and colon (*e* and *f*) mucosa, respectively. (*a*, bar = 0.21 μ m, $\times 3250$; *b* and *c*, bar = 0.25 μ m, $\times 3950$; *d*, bar = 0.32 μ m, $\times 4840$; *e* and *f*, bar = 0.25 μ m, $\times 3950$.)

increased by $\approx 70\%$ after 2 hr. The plasma GSH levels (obtained from the right ventricle) of fasted mice (for 2 days) just before and 30, 60, and 90 min after administration of GSH (4 mmol/kg) by gastric lavage were, respectively, $41.3 \pm 1.5 \mu$ M, $47.4 \pm 3.4 \mu$ M, $42.0 \pm 5.5 \mu$ M, and $39.1 \pm 12 \mu$ M, indicating no significant change. Similar results were obtained in studies on mice fed ad libitum. Fed mice were given BSO (2.5 mmol/kg p.o. and 2.5 mmol/kg i.p.) and after 90 min were given (p.o.) saline (group a), GSH (4 mmol/kg) (group b), or GSH (1 mmol/kg) (group c). The plasma GSH values for the controls were $18.3 \pm 2.4 \mu$ M and $22.5 \pm 0.3 \mu$ M for 30 and 90 min, respectively, after saline administration (group a); the corresponding values for group b were $29.6 \pm 1.8 \mu$ M and $27.0 \pm 2.6 \mu$ M; the values for group c were $39.8 \pm 4.1 \mu$ M and $18.8 \pm 2.2 \mu$ M. The findings show a transient increase in plasma GSH level after administration of GSH to BSO pretreated mice; the effect seems not to be dose dependent.

DISCUSSION

A major finding here is that the epithelial cells of the jejunum and colon are highly dependent on GSH. Since the tissues were not challenged by toxic compounds or extra oxygen, it may be concluded that GSH deficiency alone leads to the marked cellular degeneration observed. That these effects are prevented by administration of GSH ester (given p.o. or i.p.) strongly supports this conclusion.

Another finding is that oral administration of GSH increases the GSH levels of jejunal and colon mucosa and protects these tissues against BSO-induced GSH deficiency. This suggests that oral administration of GSH might be of therapeutic value in protecting the gastrointestinal epithelia against toxicity associated with inflammatory disease, ischemia, oxidative damage, chemotherapy, and radiation.

GSH administered i.p. did not significantly increase the GSH levels of the jejunal mucosa, colon mucosa, pancreas, lung (14), skeletal muscle (13), heart (13), lymphocytes (14),

Table 1. GSH levels after treatment with BSO, GSH ester, and GSH

	Jejunal mucosa, $\mu\text{mol/g}$	Jejunal mucosa mitochondria, nmol per mg of protein	Gastric mucosa, $\mu\text{mol/g}$	Colon mucosa, $\mu\text{mol/g}$	Pancreas, $\mu\text{mol/g}$	Plasma, μM
Controls	0.27 ± 0.03	1.1 ± 0.03	0.32 ± 0.02	0.07 ± 0.01	0.06 ± 0.01	0.90 ± 0.20
GSH ester (p.o.)	1.73 ± 0.04	9.6 ± 0.3	1.10 ± 0.05	0.25 ± 0.02	0.42 ± 0.07	
GSH ester (i.p.)	1.04 ± 0.05		1.16 ± 0.04	0.22 ± 0.04	0.45 ± 0.06	43.0 ± 5.0
GSH (p.o.)	0.94 ± 0.14	4.8 ± 0.3	0.60 ± 0.20	0.34 ± 0.15	0.12 ± 0.04	
GSH (i.p.)	0.12 ± 0.02	1.4 ± 0.4	0.46 ± 0.07	0.07 ± 0.02	0.08 ± 0.03	$>5000^*$
Glu, Cys, Gly (p.o.)	0.23 ± 0.12		0.36 ± 0.03	0.09 ± 0.01	0.08 ± 0.02	
Not treated with BSO	2.92 ± 0.35	8.7 ± 0.7	1.73 ± 0.29	1.70 ± 0.13	1.71 ± 0.18	58.3 ± 6.0

Mice were given BSO (as stated in Fig. 1) for 7 days together with other compounds. Groups of four to six mice were given GSH ester (0.9 ml of 160 mM) by gastric lavage (p.o.), or by i.p. injection. GSH (+ ethanol and Na_2SO_4 ; see *Materials*) and a mixture of L-glutamate, L-cysteine, glycine, ethanol, and Na_2SO_4 were given in the same way as indicated in doses of 4 mmol/kg (except cysteine was at 2.5 mmol/kg) twice daily (at 11 a.m. and 6 p.m.) for 7 days. The tissue samples were obtained at 12 noon.

*Taken from a similar experiment (table 1 in ref. 14). The values for plasma GSH were 25,100; 12,080; and 5080 μM , respectively, 50, 120, and 180 min after i.p. administration of GSH.

liver (14), or lens of BSO-treated animals (15), nor did it protect lung (14), muscle (13), colon mucosa, jejunal mucosa, or lens epithelial cells (15) from cellular damage, whereas giving GSH ester did. The utilization of extracellular GSH seems to involve degradation of GSH, transport of products, and intracellular synthesis of GSH (4, 6); studies on human lymphoid cells (32, 33), kidney (34), lung (14, 35), and bovine pulmonary artery endothelial cells (36) support this. That the tissue GSH levels of BSO-treated mice, which are very low (0.06–0.3 mM), do not increase significantly when the plasma levels of GSH are 5 mM or higher (Table 1) indicates that there is little if any transport of GSH. The small increases in tissue GSH found after untreated mice are injected i.p. with GSH may probably be ascribed to operation of the pathway indicated above (degradation, uptake, and intracellular synthesis) rather than to direct transport of intact GSH. Transport (export) of intact GSH is a property of many cells under physiological conditions in which the intracellular levels of GSH are 1–10 mM and in which there are very low ($<60 \mu\text{M}$) extracellular levels of GSH (4, 6). Although this process might theoretically be reversed in the presence of very high levels of extracellular GSH, this has not been observed. High levels of GSH accumulate in the blood plasma after inhibition of γ -glutamyltranspeptidase, and there is an impressive glutathionuria, which is not accompanied by an increase of tissue GSH levels (37).

Several mechanisms may be involved in the utilization of luminal GSH by gastrointestinal mucosal cells. Cleavage of extracellular GSH to its constituent amino acids followed by their transport and intracellular GSH synthesis does not seem to account for the findings (Table 1) but is not excluded. Possible pathways include (i) formation of γ -glutamylcyst(e)ine by carboxypeptidase-type cleavage of GSH, or by transpeptidation between GSH and cyst(e)ine, followed by transport and conversion to GSH by GSH synthetase (which is not inhibited by BSO) (38); (ii) transport of GSH; (iii) transport of γ -glutamyl-GSH followed by conversion to GSH by action of γ -glutamylcyclotransferase (39); and (iv) formation of glutamate (or γ -glutamyl amino acids) and cyst(e)inylglycine followed by their transport, hydrolysis, and intracellular GSH synthesis (if the BSO block is not complete). Intestinal protein breakdown leads to a mixture of amino acids and dipeptides plus some tripeptides (40–42). Transport of dipeptides and tripeptides into intestinal cells has been shown; the peptides are split to their constituent amino acids during or after transport. GSH, which may be considered as a tripeptide or as a derivative of a dipeptide, might be a substrate for a dipeptide carrier. Luminal GSH arises from the bile, by export from the epithelial cells, breakdown of desquamated cells, and diet. Bile contains high levels (1–6 mM) of GSH (43–45), and this could lead to high local levels

of GSH in the small intestine. In the present studies, 0.9 ml of 160 mM GSH was administered by gastric lavage; this would be diluted by the luminal contents but would be expected to be much higher than the GSH level in the diet. The present findings suggest that biliary GSH normally functions to provide GSH and thus protection for the intestinal mucosa.

Several reports have appeared on the uptake of GSH from the intestinal lumen. Transport of GSH in everted sacs of rat small intestine was found to be Na^+ independent, inhibitable by triglycine and glycyl-L-leucine, and to have properties characteristic of carrier-mediated diffusion (46, 47). Studies in which an *in situ* closed-loop vascular perfusion system was used led to the conclusion that intact GSH is transported by a Na^+ -dependent process into the mesenteric circulation (48). We found very little if any increase of the GSH level of plasma (taken from the right ventricle) after administration of GSH to untreated mice by gastric lavage.[†] Higher plasma levels of GSH might possibly be found after giving larger doses of GSH. However, the potential significance of this approach must be considered in relation to the finding that there seems to be very little transport of GSH from the plasma into tissues, even with very high plasma GSH levels. The absence of a significant increase in the GSH levels of pancreas, colon mucosa, jejunal mucosa, muscle (13), heart (13), lung (14), liver (14), and lymphocytes (14), after i.p. administration of GSH, and the finding that GSH given i.p. did not protect against cellular damage due to GSH deficiency, do not argue strongly in favor of a physiologically significant uptake pathway of GSH going from intestinal lumen to blood plasma to cells. It seems quite possible that a substantial fraction, although perhaps not all, of luminal GSH is split within the lumen and absorbed as dipeptides and free amino acids. Nevertheless, as discussed above, luminal GSH may also be taken up by the gastrointestinal epithelium. Conceivably, small amounts of GSH, as in the case of certain other peptides (41, 42), might normally be absorbed intact into the plasma.

It has been concluded (51), on the basis of studies in which cells in suspension isolated from kidney (52), intestine (53), and lung (54) were protected from destruction by *t*-butylhydroperoxide, paraquat, or menadione by adding GSH, that intact GSH is transported into these cells and that protection is dependent on transport of GSH across the basolateral cell membrane because protection was decreased by probenecid (53). However, the data do not exclude

[†]Similar results were observed in rats (49). Studies on other species would be of interest. It is not expected that GSH metabolism is qualitatively different in other mammalian species; thus, the effects of inhibition of γ -glutamyltranspeptidase by giving acivicin to mice are similar to those of a deficiency of this enzyme in humans (50).

synthesis of GSH because protection was also observed after adding the free amino acid constituents of GSH (52, 53). Although BSO and acivicin were used in these studies (52–54) to inhibit, respectively, GSH synthesis (1) and GSH breakdown (50, 55), these activities are not completely inhibited under the conditions used; studies on the extent of such reactions in these cells were not reported. The inhibitions of protection observed after adding γ -glutamylglutamate and ophthalmic acid, attributed to inhibition of GSH transport (51, 52), may also be ascribed to inhibition of γ -glutamyl-transpeptidase (see ref. 56). Apart from alternative interpretations of these *in vitro* results, the present and previous (13, 14, 37) *in vivo* studies indicate that transport of intact GSH across basolateral cell surfaces, if any, must be quite low. The reported transport of GSH into rat kidney basolateral vesicles (57) has been considered in relation to data (34) that exclude the occurrence of appreciable basolateral transport *in vivo*.

Administration of GSH, a relatively nontoxic way of supplying cysteine moieties as compared to giving cysteine, may lead to cellular protection. The available evidence indicates that protection almost always depends on the availability of intracellular GSH. Therefore, application of cellular GSH delivery agents such as GSH esters is advantageous. Administered GSH monoesters are more effective than GSH in protecting against the toxic effects of acetaminophen (16), radiation (17, 58), Cd^{2+} (22), melphalan (59), cyclophosphamide (59), HgCl_2 (60), and cisplatin (61). It is of particular interest that treatment with GSH monoester leads to significant and apparently preferential increase of mitochondrial GSH levels.

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