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Glutathione metabolism in the lung: Inhibition of its synthesis leads to lamellar body and mitochondrial defects

[lymphocytes/lung type 2 cells/buthionine sulfoximine/glutathione mono (glycyl) ester/intestine]

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

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

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ABSTRACT **Mice treated with buthionine sulfoximine, an inhibitor of glutathione synthesis, showed striking alterations of morphology of lung type 2 cell lamellar bodies (swelling and disintegration) and mitochondria (degeneration) and of lung capillary endothelial cells (mitochondrial swelling). These effects probably may be ascribed to glutathione deficiency; administration of glutathione monoester protects against them. Measurements of arteriovenous plasma glutathione levels across the lung indicate that the net uptake of glutathione by this organ is substantial. Thus, glutathione exported from the liver to the blood plasma is utilized by the lung which, like liver, kidney, and lymphocytes (and unlike skeletal muscle), exhibits a high overall rate of glutathione turnover. Intraperitoneal injection of glutathione into buthionine sulfoximine-treated mice leads to very high levels of plasma glutathione without significant increase in the glutathione levels of liver, lung, and lymphocytes; on the other hand, administration of glutathione monoester leads to markedly increased tissue and mitochondrial levels of glutathione. Administration of glutathione monoester (in contrast to glutathione) to control mice also increases mitochondrial glutathione levels. The findings indicate that glutathione is required for mitochondrial integrity and that it probably also functions in the processing and storage of surfactant in lamellar bodies. The morphological changes observed after treatment with buthionine sulfoximine and their prevention by glutathione monoester as well as findings on glutathione metabolism indicate that this tripeptide plays an important role in the lung. The previously observed failure of buthionine sulfoximine-treated mice to gain weight is mainly due to glutathione deficiency in the intestinal mucosa.**

Previous studies (1) on the effects of inhibition of glutathione (GSH) synthesis on skeletal muscle provided evidence that (*i*) GSH functions in muscle under normal conditions, (*ii*) the levels of GSH in muscle are greatly in excess of those required, and (*iii*) GSH is required for mitochondrial integrity. It was also found that administration of GSH monoester (in contrast to that of GSH) led to maintenance of a significant level of GSH in muscle mitochondria during a relatively long period of administration of buthionine sulfoximine (BSO), an inhibitor of GSH synthesis.

In the present work we have extended this approach to the lung, which like kidney and liver (and in contrast to skeletal muscle) exhibits a high rate of turnover of GSH. We found evidence of significant cellular damage to lung and to a lesser extent to lymphocytes after prolonged inhibition of GSH synthesis produced by administration of BSO. Interestingly, we found striking evidence of lamellar body damage in type 2 cells and degeneration of mitochondria in these cells and in capillary endothelial cells. These changes were prevented by administration of GSH monoester. Administration of GSH monoester prevented the marked decline in tissue GSH levels found after administration of BSO and led to levels of mitochondrial GSH in the lung and liver that were higher than those found in untreated controls.

EXPERIMENTAL PROCEDURES

Materials. Mice (male, 28–32 g, Swiss–Webster; Taconic Farms) were maintained on Purina Chow. L-Buthionine-[*S,R*]-sulfoximine (BSO) (2–4), L-buthionine sulfone (4), and GSH monoisopropyl (glycyl) ester (5) were prepared as described. Hypaque (50%) was purchased from Winthrop-Breon Labs (New York). An aqueous solution of GSH monoisopropyl ester was prepared in the $\frac{1}{2}(H_2SO_4)$ form and adjusted to pH 6.5–6.8 by cautious addition of NaOH immediately prior to use. In studies in which this compound was given, control experiments were carried out in which mice were given equimolar amounts of GSH, isopropanol, and Na_2SO_4.

Methods. BSO was administered to mice as stated below (see Figs. 1 and 2 and Table 1). L-Buthionine sulfone was given to mice in equimolar amounts in parallel studies. The tissues were obtained as follows. Mice were anesthetized by intraperitoneal injection of a mixture of xylazine (5 or 10 mg/kg of body weight) and ketamine (100 mg/kg); within 3 min, the thoracic and peritoneal cavities were opened. The lung and liver were perfused from the right ventricle with 10 ml of cold saline after clamping of the venous heart input. Perfusion was completed within 2 min as the lung turned white and the liver became light brown. The left inferior lobe of the lung was excised, rinsed with cold saline, blotted, weighed, and homogenized in 5 vol of 5% sulfosalicylic acid. A piece of the left lobe of the liver was excised and processed as described for the lung. The tissue homogenates were centrifuged for 5 min at 10,000 × *g* at 4°C, and the supernatant solutions were analyzed for GSH.

Lymphocytes were obtained from whole blood (0.7 ml) drawn from the right ventricle into a heparinized syringe containing 0.7 μmol of EDTA and 14 μmol each of L-serine and sodium borate (6). Samples obtained from two mice were pooled and mixed with 2.6 ml of cold saline and then layered on top of a Hypaque/Ficoll gradient in a plastic tube (6, 7). The gradient consisted of two phases: an upper phase containing 3 ml of a mixture of 10 parts of 33.9% Hypaque and 24 parts of 9% Ficoll, and a lower phase containing a mixture of 3 ml of 10 parts of 50% Hypaque and 30 parts of 9% Ficoll. The lymphocytes were separated from granulocytes by differential centrifugation; they were centrifuged at 300 × *g* for 30 min at 4°C rather than 800 × *g* as recommended (7) so as to decrease contamination by platelets. The lymphocyte suspensions (which contained about 10% monocytes) were washed by centrifugation with cold saline containing 1 mM EDTA, 20 mM L-serine, and 20 mM sodium borate. Contaminating erythrocytes were lysed by suspension of the cells in 0.15 mM ammonium chloride at 37°C for 5 min (6, 8). The lymphocytes

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Abbreviations: GSH, glutathione; BSO, buthionine sulfoximine.

were washed twice with saline (containing EDTA, serine, and sodium borate), counted, and lysed by two cycles of freezing/thawing in 0.1–0.2 ml of 4.3% sulfosalicylic acid as described (6, 9). After centrifugation, the supernatant solutions were immediately analyzed for total GSH.

Determinations of total GSH were also carried out on plasma obtained by immediate centrifugation of 0.2 ml of whole blood [from the right ventricle (see above) or by direct aspiration (0.05 ml) from the left ventricle] at 10,000 × *g* at 4°C for 1 min (10, 11). A portion of the clear supernatant solution was immediately mixed with sulfosalicylic acid (final concentration, 4.3%) and then centrifuged for 5 min at 4°C. The supernatant solution obtained was used for determination of total GSH. The presence of hemoglobin in the samples was examined (12, 13). The minute amount of hemoglobin found indicated that the presence of erythrocytes could account for <0.3% of the observed GSH content (14).

GSH determinations carried out as described above on control samples of lung, liver, and lymphocytes were, within experimental error, identical to values obtained on mice killed by spinal cord transection. The anesthesia procedure is advantageous in that significantly larger amounts of blood may be obtained than are obtained after spinal cord transection. However, the perfusion was completed within 5 min; after longer periods of anesthesia, even with one-third of the doses stated above, lower GSH levels were found.

Total GSH was determined by the glutathione disulfide reductase–5,5-dithiobis(2-nitrobenzoate) recycling method (11). For plasma, values that include mixed disulfides between GSH and cysteine or proteins were also determined after reduction with 5 mM dithiothreitol (9). BSO was determined by use of a Durrum (model 500) amino acid analyzer. The activities of γ-glutamylcysteine synthetase, γ-glutamyl transpeptidase, and citrate synthase (15, 16) were determined as stated (1). Protein was determined (17), and electron microscopy (1) was performed as previously stated.

The mitochondrial fractions of liver and lung were separated by the procedure previously given (1, 16, 18, 19) except that heparin was omitted in the homogenization step. The fractions obtained by this procedure were checked by electron microscopy. The fractions from liver contained virtually uncontaminated mitochondria; those from lung contained predominantly mitochondria and about 5% lamellar bodies.

RESULTS

Effect of Administration of BSO on GSH Levels. Administration of BSO led to rapid decline of the levels of GSH in the lung, lymphocytes (Fig. 1), liver, and plasma (Fig. 2). The initial rapid rates of decline, expressed as the time required for a 50% decrease in the level, were about 25 and 45 min for lymphocytes and lung, respectively. The decline in the plasma GSH level, as expected (20, 21), was closely parallel to that of the liver ($t_{1/2}$ = about 65 min). The decline of the GSH levels in the liver was biphasic; the second slower rate, as discussed previously (1, 18), is thought to reflect loss of GSH from the mitochondria. The slower later rates of GSH disappearance were 0.01%, 0.02%, 0.05%, and 0.05% of the corresponding initial rates for lymphocytes, lung, liver, and plasma, respectively. The disappearance of GSH from lymphocytes and lung exhibited both rapid and slow phases but seemed to be more complex than in liver. The data obtained on GSH levels in the lung are probably a function of several factors, including the cellular heterogeneity of this organ, the rates of cellular export of GSH, and the intracellular levels of BSO. Although relatively high overall levels of BSO were maintained in these experiments, the intracellular level of BSO in the various lung cell types is not known. BSO was rapidly cleared from plasma ($t_{1/2}$ = 30–40 min), and this compound was rapidly excreted in the urine. Preliminary studies in which single doses of BSO were administered indicate that the time required for a 50% decrease in tissue BSO level is about 40, 50, and 120 min for liver, lung, and lymphocytes, respectively. After discontinuation of treatment with BSO, the tissue levels of BSO declined rapidly, and the levels of GSH increased significantly (see Fig. 1 *Inset* and Fig. 2 *Inset*). The rates at which normal levels of GSH return were lower than the initial rates of GSH disappearance, and the GSH values "overshot" the control values as observed in the heart (see figure 1 in ref. 1).

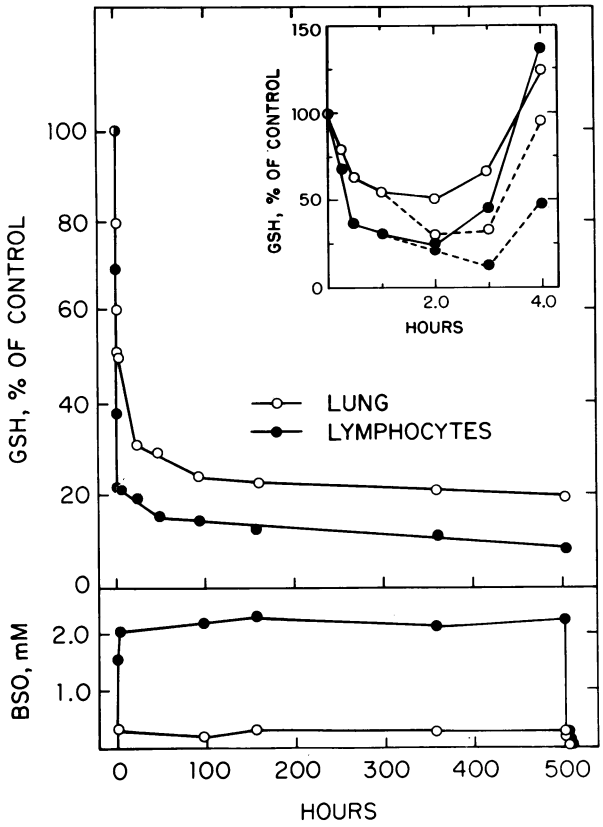

FIG. 1. Effect of treatment with BSO on the GSH level of lung and lymphocytes. Mice were given BSO twice daily by intraperitoneal injection (4 mmol/kg) at 9 a.m. and at 6 p.m. for 21 days. BSO was also given in the drinking water (20 mM). Controls were injected with an equivalent volume of saline and were given tap water to drink. Control values of GSH were 1.69 ± 0.12 μmol/g (lung) and 1.39 ± 0.15 nmol per 10^6 cells (lymphocytes). After 0.25 hr, 0.5 hr, 1 hr, 2 hr, 1 day, 2 days, 4 days, 7 days, 15 days, and 21 days of BSO treatment, the GSH values (mean ± SD; n = 3–5) for lung were, respectively, 1.31 ± 0.16, 1.04 ± 0.15, 0.92 ± 0.05, 0.91 ± 0.10, 0.54 ± 0.06, 0.47 ± 0.05, 0.43 ± 0.05, 0.42 ± 0.04, 0.39 ± 0.04, and 0.38 ± 0.04 μmol/g. The corresponding values for lymphocytes were 0.97 ± 0.11, 0.51 ± 0.08, 0.42 ± 0.05, 0.36 ± 0.05, 0.27 ± 0.04, 0.25 ± 0.04, 0.24 ± 0.04, 0.18 ± 0.02, 0.16 ± 0.02, and 0.14 ± 0.03 nmol per 10^6 cells. (*Inset*) Effect on lung (○) and lymphocyte (●) GSH levels of a single injection (—) and of two injections (---) of BSO (at 0 and 1 hr; each dose, 8 mmol/kg).

The rapid rates at which GSH levels increased after discontinuation of BSO treatment precluded studies on the effects of amino acid supplementation on BSO-treated mice, as was done in studies on muscle (1). However, studies on fed and fasted mice showed that administration either of cysteine or of a mixture of cysteine, glycine, and glutamate increased the GSH levels of lung, liver, and lymphocytes to about the same extent. Administration of glutamate (or of α-ketoglutarate) alone also increased liver and lung GSH levels in fed mice. Administration of glycine or glutamine did not increase GSH levels in these tissues or in lymphocytes.

The γ-glutamylcysteine synthetase activities of lymphocytes and lung were found to be 87.0 ± 4.8 (mean ± SD; n = 5) and 10.3 ± 0.1 nmol/hr per mg of protein, respectively; the value for liver is 82.3 ± 1.5 nmol/hr per mg of protein (18).

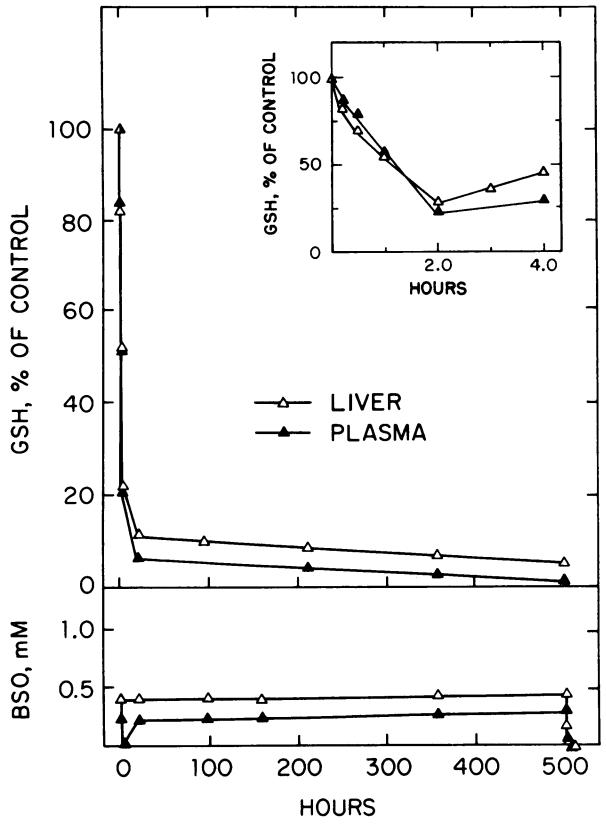

FIG. 2. Effect of a single dose (*Inset*) or multiple doses of BSO at 9 a.m. and 6 p.m. (each dose, 2 mmol/kg) on GSH levels of liver (△) and plasma (▲). Control values of GSH were 8.26 ± 0.06 μmol/g (liver) and 58.3 ± 4.8 μM (venous plasma from right ventricle). After 0.25 hr, 0.5 hr, 1 hr, 2 hr, 1 day, 4 days (only liver), 9 days, 15 days, and 21 days of BSO treatment, the GSH values (mean ± SD; $n = 3$–5) for liver were, respectively, 6.61 ± 0.28, 5.78 ± 0.25, 4.54 ± 0.21, 2.48 ± 0.09, 1.10 ± 0.08, 0.83 ± 0.05, 0.66 ± 0.04, 0.59 ± 0.03, and 0.52 ± 0.02 μmol/g. The corresponding GSH values for plasma were 50.7 ± 2.9, 46.6 ± 3.1, 33.2 ± 3.2, 12.9 ± 1.5, 3.50 ± 0.50, 2.92 ± 0.3, 1.80 ± 0.3, and 1.20 ± 0.2 μM. A plasma BSO value of close to zero was found at 2 hr.

The γ-glutamyl transpeptidase activities of lymphocytes, lung, kidney, and liver are, respectively, 41.2 ± 1.2 (mean ± SD; $n = 5$), 25.0 ± 0.3, 26,300 ± 3700 (22), and 1.92 ± 0.05 nmol/hr per mg of protein (18); see also refs. 20 and 23–27.

Utilization of GSH by the Lung. Previous studies on nephrectomized mice led to the conclusion that about two-thirds of the arterial blood plasma GSH is used by the kidney and that about one-third of the plasma GSH is used by extrarenal γ-glutamyl transpeptidase activity (21). On the basis of studies on the isolated perfused rat lung, Berggren *et al.* (24) concluded that the lung in addition to the kidney may utilize plasma GSH and that utilization of GSH is probably mediated by extracellular breakdown and resynthesis rather than by direct uptake. The present studies, which lead to similar conclusions, make it possible to consider a quantitative estimate of the amount of GSH used by the lung; interestingly, the lungs use more GSH than do the kidneys.

Plasma from blood drawn from the right ventricle has GSH levels of 58.3 ± 4.8 μM (Fig. 1), and levels that include GSH mixed disulfides of 62.1 ± 5.0 μM. In contrast, GSH levels of plasma taken from the left ventricle were found to be 22.1 ± 2.3 μM, and levels that include GSH mixed disulfides of 29.9 ± 7.5 μM. The arteriovenous difference, about 32 nmol/ml, when multiplied by the cardiac output [≈6.3 ml of plasma per min (28)], indicates that the loss of GSH from plasma during transit through the lung is about 200 nmol per min. This value is greater than has been estimated for utilization of GSH by mouse kidney (about 50 nmol/min) (29).‡

Morphological Changes Associated with Administration of BSO. Electron microscopic studies of the liver and kidney after 3 weeks of treatment with BSO showed no abnormalities. In contrast, striking changes were noted in the lung type 2 cells and lung capillary endothelial cells (Fig. 3). In type 2 cells there was a significant decrease in the number of mitochondria and evidence of mitochondrial swelling and degeneration. After treatment with BSO the lamellar bodies seemed to dominate the type 2 cells; the lamellar bodies became larger, less dense, and appeared to occupy more space than in the controls. Swelling of the lamellar bodies was associated with disruption of the characteristic lattice structure of these organelles. There was a decrease in the number of microvilli on the alveolar surface of the type 2 cells, swelling and thickening of the basal membrane, and swelling of mitochondria in the lung capillary endothelial cells. Blunting of the microvilli of the lymphocytes was also seen. These changes in morphology were not seen in mice given buthionine sulfone nor were they found in mice treated with BSO and GSH monoester. They were found in mice treated with BSO and GSH. Determinations of citrate synthase activity were carried out on the mitochondrial fraction obtained from lung; after 9 days of treatment with BSO, this activity was 0.025 ± 0.003 μmol/min per mg of protein as compared with values of 0.094 ± 0.007 for controls. Mice treated with BSO for 3 weeks and then allowed to recover for 8 weeks did not show the morphological changes described above; the citrate synthase activity of the mitochondrial fraction of lung was about the same as that of untreated controls.

Effect of Administration of GSH Monoester and of GSH on GSH Levels. When mice were treated with BSO for 9 days, there was as expected a marked decline in the levels of cellular GSH and of plasma GSH (Table 1, experiment 2 vs. experiment 1). When GSH monoester was given together with BSO (Table 1, experiment 3), the liver GSH levels were about the same as the levels in controls, and the GSH level of liver mitochondria was somewhat greater than the levels in controls. Administration of GSH monoester led to higher levels of GSH in the lung (experiment 3 vs. experiment 2); the mitochondrial fraction obtained from lung had GSH levels that were higher than those in the controls (experiment 3 vs. experiment 1). The GSH values for lymphocytes treated with BSO and GSH monoester were higher than those found for the BSO-treated mice (experiment 3 vs. experiment 2). When GSH was given together with BSO (experiment 4), very high plasma levels of GSH were found (about 80 times greater than the control values), but there was only a slight increase (perhaps within the limits of error) of the GSH levels of liver and lung, of the mitochondrial fractions obtained from these tissues, and of the lymphocytes. As shown previously, administration of GSH to untreated mice has only a slight effect or no effect on liver and kidney GSH levels (32). Similar findings were made in the present studies on lung; for example, untreated mice injected with GSH (5 mmol/kg) exhibited lung GSH levels that were only 30% greater than those of controls after 2 hr and were about the same as the controls after 4 hr. Administration of GSH monoester to untreated mice led to levels of liver mitochondrial GSH that were significantly higher (≈120%) than those found in controls, whereas administration of equimolar amounts of GSH did not affect liver mitochondrial GSH levels.

‡The arteriovenous difference in plasma GSH level across the rat lung is smaller than was found here for the mouse because there is a lower plasma level of GSH in the rat right ventricle than in that of the mouse. Estimates of GSH utilization by rat lung and kidney (30) based on arteriovenous differences in plasma GSH levels across these organs and cardiac output (31) indicate that kidney and lung are both highly active in utilization of plasma GSH.

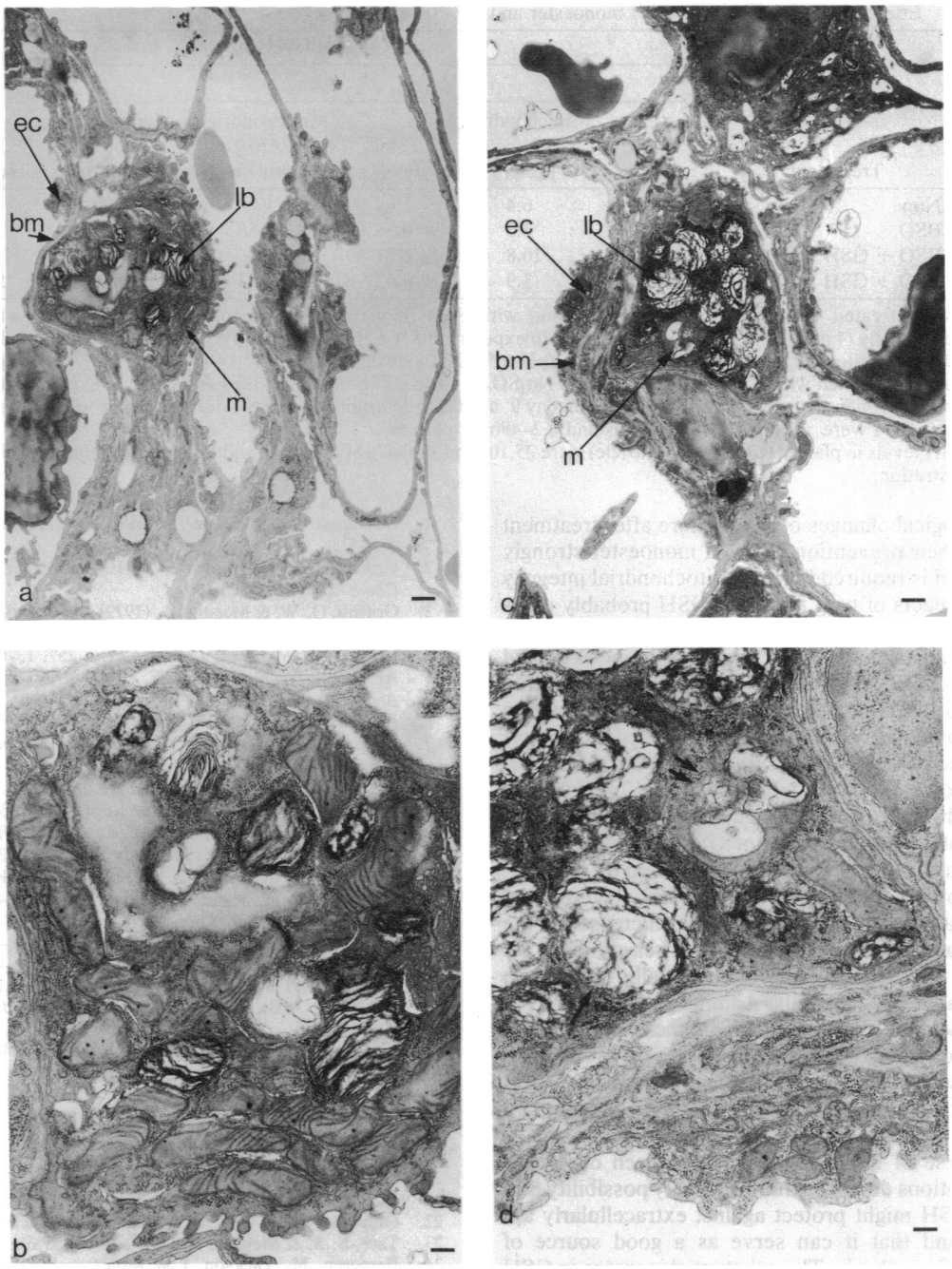

FIG. 3. Representative electron micrographs of the lungs of control mice (*a* and *b*) and mice treated with BSO for 21 days (*c* and *d*). *c* and *d* show lamellar body (lb) swelling and disintegration (one arrow), mitochondrial (m) degeneration (2 arrows), endothelial cell (ec) mitochondrial swelling, and thickening of the basal membrane (bm). [*a* and *c* = ×5400 (horizontal bar in the lower right corner = 0.60 μm); *b* and *d* = ×17,800 (horizontal bar in the lower right corner = 0.18 μm).]

DISCUSSION

The production of cellular GSH deficiency by treatment with BSO has advantages over various methods of GSH depletion (compare ref. 20). Thus, decreased GSH levels can be maintained for relatively long periods, facilitating detection of structural and other abnormalities. It seems significant that the effects of BSO on tissue and mitochondrial GSH levels, skeletal muscle degeneration (1), morphological changes in the lung, and depression of weight gain (1, §) are virtually completely prevented by giving GSH monoester but not by giving GSH. Although BSO is highly effective as an inhibitor, relatively high doses must be given to maintain effective cellular levels, and BSO is only slowly transported into some organelles [e.g., mitochondria (18)] and tissues [e.g., brain (20, 21)].

§The effect of BSO on weight gain noted previously (1) was confirmed in the present studies; this effect is largely prevented by administration of GSH monoester but is not affected by administration of GSH. The finding that mice given BSO develop diarrhea led us to examine the effect of BSO administration on intestinal GSH levels. After 14 days of treatment with BSO (oral and intraperitoneal), the levels of GSH in mouse jejunal mucosa, colon mucosa, and pancreas were about 4% of the controls. Electron microscopy showed microvillus and mitochondrial degeneration and vacuolization of the epithelial cells of jejunal mucosa. Previous studies showed that rat jejunal mucosal cells have high levels of GSH (33). It is probable that the normal absorptive functions of the gut require GSH.

Table 1. Effect of administration of GSH monoester and of GSH on GSH levels

		GSH levels					
		Liver		Lung			
Exp.	Treatment*	Total, μmol/g	Mitochondrial, nmol/mg of protein	Total, μmol/g	Mitochondrial, nmol/mg of protein	Lymphocyte total, nmol per 10^6 cells	Plasma total, μM
1	None	8.26	6.4	1.69	4.7	1.39	58.3
2	BSO	0.98	3.8	0.36	0.86	0.20	0.90
3	BSO + GSH monoester	8.29	10.8	0.71	10.0	0.45	43.0
4	BSO + GSH	1.10	3.9	0.42	0.87	0.24	5080†

*Mice were treated with saline (experiment 1) and with BSO (experiments 2–4) for 9 days. The BSO was given intraperitoneally (4 mmol/kg) at 10 a.m. and 6 p.m. In experiment 3, GSH monoisopropyl ester (5 mmol/kg; 1–1.3 ml) was injected intraperitoneally as an isosmolar solution (pH 6.5–6.8) twice daily (8 a.m. and 4 p.m.) for 9 days. In experiment 4, mice were injected with GSH, isopropanol, and Na_2SO_4 in amounts equimolar to the ester given in experiment 3. Mice (4–5 per experiment) were sacrificed at 11 a.m. on day 9, and GSH determinations were done as described. Data are given as means; SDs were ± 0.04–0.08 for tissues and 0.2–480 for plasma.

†The GSH levels in plasma (from right ventricle) were 25,100 and 12,080 μM at 50 min and 120 min, respectively, after GSH administration.

The morphological changes observed here after treatment with BSO and their prevention by GSH monoester strongly suggest that GSH is required for lung mitochondrial integrity and for other aspects of lung function. GSH probably plays a role in the processing and storage of lung surfactant. The high level of GSH in the alveolar epithelial lining fluid and related studies on this interesting finding (34) suggest that surfactant and GSH are of major importance in the protection of the lung against toxic compounds in the inhaled air. Export of GSH from such cells as phagocytes, type 2 cells, lymphocytes, and fibroblasts may contribute to the GSH of the alveolar lining fluid. The present studies reveal an additional aspect of the interorgan transport of GSH in which plasma GSH (whose major source is the liver) is utilized by the lung.

Administration of GSH led to extraordinarily high plasma GSH levels (Table 1) but not to significant increases in the GSH levels of lung, liver, or lymphocytes. These findings are in accord with the view that there is normally little or no transport of intact GSH into cells. Administration of GSH to control mice does not lead to appreciable increases in tissue GSH levels, and the small increases that are observed most likely may be ascribed to extracellular degradation, transport of the products, and intracellular synthesis of GSH (compare ref. 20). Under physiological conditions many cells export GSH; the reverse of this process has not been observed. These considerations do not exclude the likely possibility that administered GSH might protect against extracellularly applied toxicity and that it can serve as a good source of cysteine for GSH synthesis. The substantial increase in GSH levels found after administration of GSH monoester confirms that this compound is well transported and converted intracellularly to GSH (5, 32). The high levels of GSH found in the mitochondrial fractions of lung and liver after administration of GSH monoester suggest that this agent may be useful for protection against various types of toxicity.

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