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Mitochondrial damage in muscle occurs after marked depletion of glutathione and is prevented by giving glutathione monoester

[heart/glutathione mono(glycyl)ester/buthionine sulfoximine]

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ABSTRACT **Skeletal muscle degeneration associated with mitochondrial damage was found after marked depletion of glutathione produced by administration to mice of buthionine sulfoximine, an irreversible inhibitor of γ-glutamylcysteine synthetase. No mitochondrial damage was found in heart. These studies show that in the absence of applied stress (such as ischemia, drug toxicity), very marked depletion (to ≈3% of the controls) of glutathione must occur before skeletal muscle mitochondria are affected and thus suggest that muscle has a large excess of glutathione. Depletion of glutathione followed a biphasic pattern in skeletal muscle and heart, probably reflecting, in the slow phase, loss of glutathione from mitochondria. Skeletal muscle degeneration did not occur when glutathione monoisopropyl ester was given together with buthionine sulfoximine; it did occur, however, when glutathione was given together with buthionine sulfoximine. Administration of the glutathione monoester (but not of glutathione) prevented the marked decline of mitochondrial glutathione produced by buthionine sulfoximine in skeletal muscle and increased the level of glutathione in heart mitochondria to values higher than the controls. The findings suggest that glutathione monoesters may be useful agents for protection of heart and skeletal muscle against toxicity.**

A number of studies have been carried out on the metabolism and turnover of glutathione (GSH) in liver, kidney, other tissues, and various cells grown in culture (1, 2). The studies described here were undertaken to examine the GSH status of skeletal and cardiac muscle. A major fraction of total body GSH is found in skeletal muscle. The approach described previously (3, 4) was used, in which experimental animals are treated with buthionine sulfoximine (BSO), an irreversible inhibitor of γ-glutamylcysteine synthetase (3). Inhibition of this enzyme, which catalyzes the first step in GSH biosynthesis, decreases cellular levels of GSH because GSH continues to be exported and utilized without significant resynthesis of GSH.

In the course of these studies, we unexpectedly found striking evidence of skeletal muscle degeneration in mice that had been treated with buthionine sulfoximine for 2–3 weeks. Muscle degeneration, which was associated with mitochondrial damage, is apparently reversible. Muscle degeneration was not found after administration of buthionine sulfone, an analog of buthionine sulfoximine that does not inactivate γ-glutamylcysteine synthetase (5). Muscle degeneration and mitochondrial damage associated with administration of buthionine sulfoximine were prevented by simultaneous administration of GSH monoisopropyl ester (but not of GSH).

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EXPERIMENTAL PROCEDURES

Materials. Mice (male, 28–32 g, Swiss-Webster; Taconic Farms) were maintained on Purina Chow ad libitum. BSO (3, 5), L-buthionine sulfone (5) and L-γ-glutamyl-L-α-aminobutyrate (6) were prepared as described.

GSH monoisopropyl(glycyl) ester (7) was prepared in the ½(H_2SO_4) form; an aqueous solution of the ester was adjusted to pH 6.5–6.8 by cautious addition of NaOH just prior to use. An equivalent quantity of Na_2SO_4 was injected (together with GSH and isopropanol) in the control studies. γ-Glutamyl cyclotransferase was purified from sheep brain acetone powder (8). L-[U-^{14}C]Glutamate was obtained from New England Nuclear and purified by Dowex 50 chromatography just before use. The amino acids, sodium α-ketoglutarate, 5,5′-dithiobis(2-nitrobenzoate), and the other enzymes used for analytical purposes were obtained from Sigma.

Methods. BSO was given by intraperitoneal injection at 9 a.m. and 5:30 p.m. (2 mmol/kg; 0.4–0.5 ml), and in the drinking water (20 mM) (Fig. 1, Table 1). Controls were given tap water to drink and were injected with an equivalent volume of saline. In parallel studies, mice were treated with buthionine sulfone in equimolar amounts.

The mice were killed by spinal cord transection. The heart was perfused with cold saline *in situ* by inserting a syringe (butterfly 23 × 3/4; 12-inch tubing) into the right ventricle. The venus input was clamped and the perfusion procedure was repeated for the left ventricle and terminated after the coronary vessels became pale. Perfusion was performed in <10 sec; longer periods of perfusion led to significant decreases in GSH levels. The heart was excised, opened, rinsed with cold saline, blotted, weighed, and homogenized in 5 vol of 5% (wt/vol) 5-sulfosalicylic acid. The entire procedure was carried out within 2–3 min. The homogenate was centrifuged (10,000 × *g*, 5 min; Beckman Microfuge), and the supernatant solution was immediately processed for the determination of GSH; when stored for 4 days at −20°C, there was about a 30% decrease in GSH level. Skeletal muscle samples from the superficial part of the quadriceps were freed from connective tissue and fat, dipped twice in cold saline, blotted, weighed, and homogenized in 5 vol of 5% sulfosalicylic acid.

Total GSH was determined by the glutathione disulfide reductase–5,5′-dithiobis(2-nitrobenzoate) recycling method (9). BSO was determined by use of a Durrum (model 500) amino acid analyzer. γ-Glutamylcysteine synthetase activity was determined by following the rate of formation of 5-oxo[^{14}C]proline from L-[^{14}C]glutamate in the presence of excess γ-glutamyl/cyclotransferase and a creatine kinase–phosphocreatine ATP-regenerating system (10, 11). The reaction was initiated by adding 40–60 μl of the centrifuged homogenate. The tissues were homogenized (1:2.5, wt/vol)

Abbreviations: GSH, glutathione; BSO, buthionine sulfoximine.

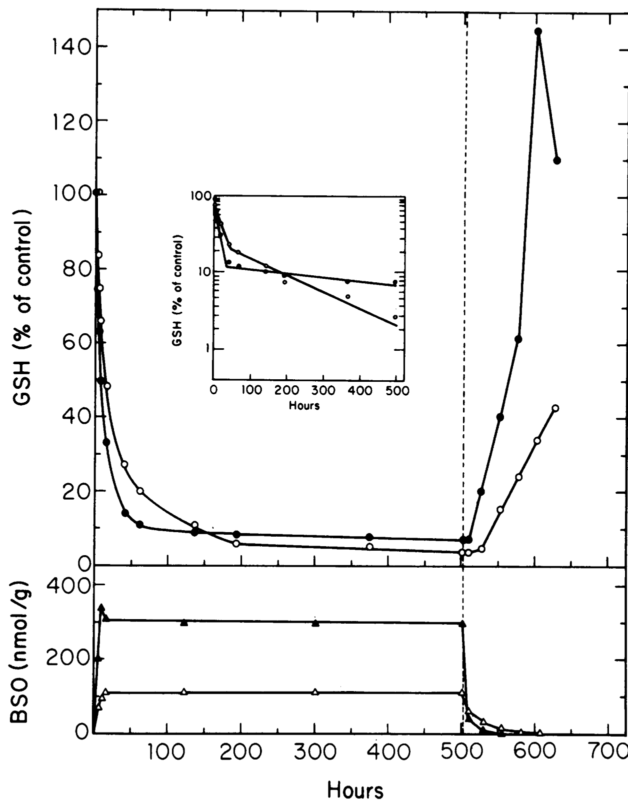

FIG. 1. Effect of BSO treatment on heart (solid symbols) and skeletal muscle (open symbols). Mice were injected intraperitoneally with BSO twice daily and given BSO solution in place of drinking water. Determinations of GSH and BSO were done as described. Control (untreated mice) values of GSH were 1.15 ± 0.071 (heart) and 0.77 ± 0.053 (skeletal muscle) μmol/g. After 6 hr, 1 day, 7 days, 14 days, and 21 days of BSO treatment, the GSH values (mean ± SD; n = 3–5) for heart were, respectively, 0.65 ± 0.051, 0.32 ± 0.025, 0.11 ± 0.015, 0.092 ± 0.017, and 0.084 ± 0.005. The corresponding values for skeletal muscle were 0.51 ± 0.06, 0.32 ± 0.027, 0.069 ± 0.015, 0.054 ± 0.004, and 0.023 ± 0.003. (*Inset*) Logarithmic plot of the data.

in 5 mM Hepes buffer (pH 7.4) containing 150 mM KCl, 0.1 mM EDTA, and 100 units of heparin per ml; the homogenates were centrifuged at 30,000 × g for 20 min. The assay mixtures were supplemented with 1 mM aminoxyacetate, a transaminase inhibitor that does not affect the activity of γ-glutamylcysteine synthetase (12). Assays were carried out in the presence and absence of BSO; the reported values are based on the BSO-inhibitable activity. Hemoglobin was determined on the homogenates of skeletal muscle and heart (13); from the values obtained, it was calculated that erythrocyte contamination could have been responsible for at most only 0.5% of the γ-glutamylcysteine synthetase activity and <0.3% of the observed GSH values (cf. refs. 14 and 15). Protein was determined by the Bradford (16) procedure using bovine serum albumin as the standard. γ-Glutamyl transpeptidase was determined at 37°C on the tissue pellet using γ-glutamyl p-nitroanilide and glycylglycine as substrates (17) in the presence and absence of 20 mM L-serine and 20 mM sodium borate. A suspension of the pellet, which was obtained by centrifugation at 18,000 × g of the homogenate [prepared in 50 mM Tris·HCl (pH 8.0), containing 150 mM KCl, 0.1 mM EDTA, and 100 units of heparin per ml], was added to initiate the reaction. After 60 min, the formation of p-nitroaniline was determined; the reported values are based on serine–borate inhibitable activity.

For electron microscopy, 10–15 ml of 4% glutaraldehyde/4% paraformaldehyde in 0.1 mM sodium phosphate buffer (pH 7.4) containing sufficient NaCl to bring the final mixture to 300 mosM was perfused through the heart for 3–5 min. Sections of skeletal muscle and heart were placed in 2% glutaraldehyde/2% paraformaldehyde for at least 2 hr at 4°C. After washing three times with the same buffer, the tissue was fixed for 1 hr in 1% OsO_4 in 0.05 M sodium phosphate buffer (pH 7.4). The tissue was dehydrated in a graded manner with ethanol, transferred to propylene oxide, and embedded in Epon 812. Sections (50–80 nm) were stained with uranyl acetate and lead citrate and observed in a JEOL 100 CX II electron microscope at 80 kV. Magnifications were calibrated with a negatively stained catalase standard (18).

Citrate synthase activity was determined after homogenization of skeletal muscle in 10 vol of 5 mM Hepes buffer (pH 7.4) containing 70 mM sucrose, 200 mM mannitol, and 100 units of heparin per ml. Homogenization was carried out for 20–30 sec with two strokes using a Teflon piston at low speed; the homogenate was centrifuged (600 × g, 5 min, 2°C). After addition of 0.5% (wt/vol) bovine serum albumin to stabilize the mitochondrial membranes (19), the supernatant obtained was centrifuged at 7000 × g for 10 min at 2°C. Enzyme activity of the mitochondria was measured after sonication (19) as described (20); in this procedure, formation of CoASH is followed by reactions with 5,5′-dithiobis(2-nitrobenzoate). GSH determinations were carried out on the mitochondria after two cycles of freezing and thawing in 5 vol of 5% sulfosalicylic acid and after centrifugation for 5 min at 10,000 × g (4°C).

RESULTS

GSH Turnover Studies. When mice were treated with BSO, the level of GSH in the skeletal muscle declined steadily, reaching a value after 200 hr that was close to 5% of the control. The decline was biphasic; the fast and slow components exhibited $t_{1/2}$ values of 16 and 120 hr, respectively (Fig. 1 *Inset*). Similar data were found for GSH levels of the heart; the fast and slow components had $t_{1/2}$ values of 5.5 and 460 hr, respectively. After 500 hr, skeletal muscle and heart had GSH levels that were, respectively, about 3% and 8% of the

Table 1. Effect of amino acid supplementation on GSH levels

		Skeletal muscle, μmol/g			Heart, μmol/g		
Exp.	Treatment	Fed	Fasted	BSO	Fed	Fasted	BSO
1	None (controls)	0.78	0.67	0.19	1.18	0.91	0.40
2	Cysteine	0.93	0.80	0.47	1.43	1.16	1.30
3	Cysteine + glutamate + glycine	0.94	0.81	0.50	1.44	1.18	1.32
4	Glutamate	0.86	0.77	0.42	1.23	1.06	0.98

The mice were fed ad libitum, fasted (96 hr), or treated with BSO (as in Fig. 1; see *Methods*). The additional compounds were administered by intraperitoneal injection (2 mmol/kg; isosmolar solution at 9 a.m.). Analyses for GSH were done at 11 a.m.; this was found to be the time interval at which maximum increase occurred. For the BSO-treated mice, the amino acids were given 2 days (heart) and 5 days (skeletal muscle) after discontinuation of BSO treatment. The controls were time-matched with the treated mice. Groups consisted of 4 or 5 mice; data are given as means (SD was ±0.01 to ±0.05).

controls. These tissues contained substantial levels of BSO during the period of treatment—i.e., ≈300 nmol/g for heart and ≈100 nmol/g for skeletal muscle. [These levels are similar to those found to deplete GSH in *in vitro* studies on primary cultures of heart cells (21).] After discontinuation of BSO treatment at 500 hr, the levels of BSO declined rapidly (more rapidly in heart) and the levels of GSH increased. In skeletal muscle the GSH level increased linearly at a rate that was significantly lower than that of initial GSH depletion. The return of GSH was more rapid in heart, to values above the initial ones. Control levels of GSH were found 5 days after discontinuation of BSO treatment in the heart, at which time only 40% of the control levels of skeletal muscle GSH had been restored.

The relatively slow rate of increase of the GSH levels, as compared to those of the initial rates of GSH depletion, may probably be ascribed to decreased γ-glutamylcysteine synthetase activity and to low concentrations of substrates. Mice depleted of GSH as described in Fig. 1 were then separately treated, after discontinuation of BSO treatment (and in the absence of tissue BSO), with cysteine, glutamate, α-ketoglutarate, glutamine, or glycine, and also with a mixture containing cysteine, glutamate, and glycine (Table 1). Fasted animals and animals fed ad libitum were also studied. Administration of cysteine or of a mixture containing cysteine, glutamate, and glycine increased the GSH levels significantly in all groups. There was some increase after administration of glutamate or α-ketoglutarate, but none was found after giving glutamine or glycine. The collected findings indicate that synthesis of tissue GSH under these conditions is mainly limited by the availability of cysteine.

Low but significant levels of γ-glutamylcysteine synthetase were found in heart [13.9 ± 1.1 (SD) nmol per hr per mg of protein] and skeletal muscle (5.5 ± 0.5 nmol per hr per mg of protein); under comparable conditions, the value found in liver was 78 ± 2.8 nmol per hr per mg of protein; this value is similar to that reported (12). Very low activities of γ-glutamyl transpeptidase were found in both heart (0.84 ± 0.05 nmol per hr per mg of protein) and skeletal muscle (0.72 ± 0.03 nmol per hr per mg of protein). Mouse liver γ-glutamyl transpeptidase activity determined under the same conditions was 12.4 ± 0.8 nmol per hr per mg of protein, a value similar to that previously found (12).

Mitochondrial Damage After GSH Depletion. Gross examination of the skeletal muscle of mice treated with BSO for 2–3 weeks showed a number of white patches; these were scattered and found mainly on the anterior and lateral sides of the legs, buttocks, and back and on the lateral sides of the trunk. Light microscopic examination showed myofiber necrosis with leukocyte and macrophage infiltration. Electron microscopy showed evidence of mitochondrial swelling and vacuolization with rupture of cristae and mitochondrial membrane disintegration (Fig. 2). Both subsarcolemmal and intermyofibrillar mitochondria were affected. The mitochondrial changes are similar to those found after ischemia (22–24). These changes were more pronounced 2–3 days after discontinuation of BSO treatment than on the last day (21st or 24th) of treatment. Such effects on mitochondria were not observed in the skeletal muscles of mice treated with buthionine sulfone in place of BSO, nor were they observed in studies of the heart in which BSO was given. The effects of BSO treatment on skeletal muscle are apparently reversible. Thus, mice that had been treated with BSO for 3 weeks and then allowed to recover for 8 weeks were found to have no evidence of muscle damage.

The microscopic studies were supplemented by determinations of mitochondrial citrate synthase activity. Skeletal muscle mitochondrial citrate synthase activities decrease (≈80%) from the control levels (0.24 ± 0.03 μmol per min per mg of protein) to values of 0.047 ± 0.009 after 9 days of

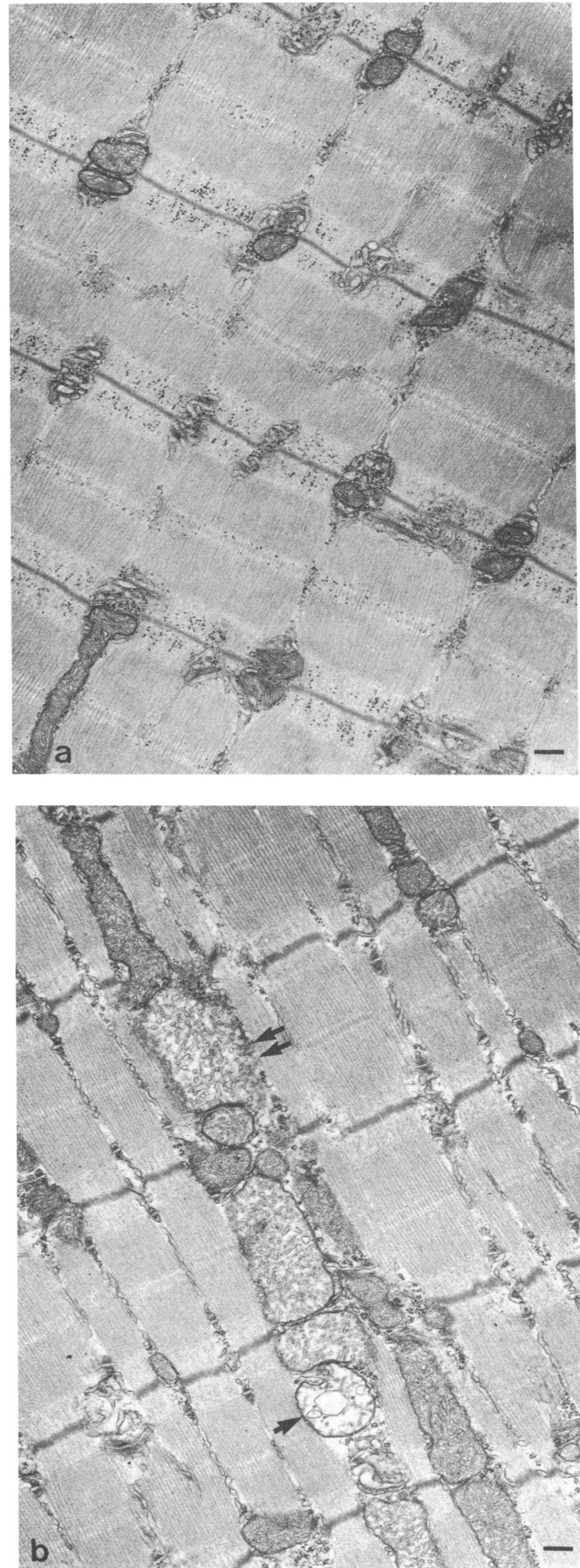

FIG. 2. Electron microscopy of skeletal muscle of control (*a*), and of BSO-treated mice (3 weeks) (*b*). Arrows in *b* indicate mitochondrial swelling, vacuolization, cristae destruction, and membrane disintegration. (×16,500; bar = 0.25 μm.)

Table 2. Effect of GSH monoester on GSH levels

		Skeletal muscle		Heart	
Exp.	Treatment	Total, μmol/g	Mitochondria, nmol per mg of protein	Total, μmol/g	Mitochondria, nmol per mg of protein
1	None (controls)	0.77 ± 0.053	5.7 ± 0.3	1.15 ± 0.071	11.5 ± 0.4
2	BSO	0.064 ± 0.01	1.2 ± 0.2	0.10 ± 0.01	5.0 ± 0.5
3	BSO + GSH + 2-propanol	0.08 ± 0.01	1.5 ± 0.2	0.12 ± 0.01	7.0 ± 0.6
4	BSO + GSH isopropyl ester	0.15 ± 0.02	5.2 ± 0.4	0.30 ± 0.20	17.4 ± 0.9
5	BSO + GSH isopropyl ester + GSH isopropyl ester (3 days)	0.20 ± 0.03	5.6 ± 0.3	0.92 ± 0.04	33.7 ± 1.2

The mice (Exps. 2–5) were treated with BSO (as in Fig. 1; see *Methods*) for 9 days. In Exps. 4 and 5, GSH monoisopropyl ester (5 mmol/kg; 1–1.3 ml) was injected intraperitoneally in an isosmolar solution (pH 6.5–6.8) twice daily (9 a.m. and 6 p.m.) for 9 days; in Exp. 5, mice were given the ester for 12 days. In Exp. 3, mice were injected with GSH and isopropanol in amounts equimolar to the ester given in Exp. 4. Mice (four or five per experiment) were sacrificed at 1 p.m. on the 9th day (12th day in Exp. 5) and GSH determinations were done as described in *Methods*. Data are given as means ± SD.

treatment with BSO. Mitochondrial citrate synthase activities were 0.30 ± 0.03 μmol per min per mg of protein 4 weeks after completion of a 3-week treatment with BSO.

Effect of Administration of GSH Monoisopropyl Ester on Mitochondrial Damage and GSH Levels. Data on the GSH levels of skeletal muscle and heart and of the mitochondrial fractions of these tissues are given in Table 2. Treatment with BSO for 9 days led to marked decreases in GSH levels (Exp. 2 versus Exp. 1). Simultaneous administration of BSO, GSH, and isopropanol led to very slightly higher GSH levels (Exp. 3). However, when BSO and GSH monoisopropyl ester were given, significantly higher GSH values were found (Exp. 4). The levels of GSH in skeletal muscle mitochondria (Exp. 4) were much higher than the corresponding values obtained in Exps. 2 and 3 and were close to those of untreated controls (Exp. 1). Similar effects were observed in the heart; here the levels of mitochondrial GSH found after GSH ester treatment (Exp. 4) exceeded those found in the controls (Exp. 1). When mice given BSO and GSH monoisopropyl ester for 9 days were then given the ester for an additional 3-day period (Exp. 5), the levels of GSH in heart mitochondria were ≈3 times greater than those of the controls. The total levels of GSH in skeletal muscle and heart were 20% and 26%, respectively, of the controls after 9 days of treatment with BSO and GSH monoester (Exp. 4).

Notably, when mice were treated for 3 weeks with BSO (as in Fig. 1) and also with two daily doses (each 5 mmol/kg) of GSH monoisopropyl ester, they did not develop white patches in their skeletal muscle; microscopic examination did not reveal evidence of muscle degeneration similar to that found after treatment with BSO alone. In contrast, mice treated with BSO + GSH + isopropanol developed muscle degeneration. When the amount of GSH monoisopropyl ester administered was decreased by 50%—i.e., to a single dose of 5 mmol/kg—there was some muscle degeneration; thus, under these conditions, complete protection required two doses (i.e., a total of 10 mmol/kg) of the ester per day.

In the course of these studies, we found that the control mice gained more weight than did mice treated with BSO. In the experiments described in Fig. 1, the control mice gained 7.5 g after 21 days, whereas mice treated with BSO did not change in weight, and the fur of these mice became shaggy. Mice given buthionine sulfone for 21 days gained ≈5.5 g. It is of interest that mice treated with BSO and GSH monoisopropyl ester showed less depression of weight gain than did mice treated only with BSO, and their fur appeared normal. Thus, in the 9-day experiment described in Table 2, the controls (Exp. 1) gained an average of 3 g, the BSO-treated mice (Exp. 2) gained an average of 0.2 g, the mice treated with BSO + GSH + isopropanol gained 1 g, and mice treated with BSO + GSH monoisopropyl ester gained 2.7 g. In another 21-day study, the observed average weight gains (given in parentheses) were as follows: controls (7.5 g), BSO-treated (0.2 g), buthionine sulfone-treated (5.5 g), BSO + GSH + isopropanol-treated (1.0 g), and BSO + GSH monoisopropyl ester-treated (6.0 g).

DISCUSSION

GSH turns over at substantial rates in skeletal muscle and heart, but the rates are lower than those found in kidney and liver (4, 25). Repeated administration of BSO gradually decreases GSH levels of heart and skeletal muscle to very low values. The biphasic decline of GSH in both tissues probably reflects sequestration of GSH in mitochondria as has been shown for liver (12). The rates at which GSH returns after discontinuation of BSO treatment are less than the initial rates of decline, and seem to reflect a relative deficiency of cysteine and possibly of glutamate. Under physiological conditions, it seems likely that plasma cyst(e)ine [and possibly γ-glutamylcysteine (26)] is a major source of cysteine for GSH synthesis in muscle and heart.

Although fasting leads to some decline of GSH levels, it is unlikely that decreased dietary intake is a major factor in depressing GSH levels and in producing myofiber degeneration in the present studies. Chronic dietary restriction in rats apparently affects few muscle fibers adversely and most of the quadriceps fibers are structurally similar to age controls (27, 28). Possibly BSO has other effects in addition to inactivation of γ-glutamylcysteine synthetase; however, it is likely that depletion of GSH *per se* affects weight gain. Since mice given both BSO and GSH monoisopropyl ester showed a gain in weight that was 80–90% of the controls, it appears that most of the effect of BSO on weight gain may be ascribed to GSH-dependent phenomena.

The finding of skeletal muscle degeneration during prolonged GSH depletion has apparently not been observed previously. Two patients with inborn γ-glutamylcysteine synthetase deficiency exhibited clinical signs of myopathy, but microscopic examination of a muscle biopsy did not show evidence of muscle degeneration (29). Newborn mice treated with BSO have been reported to develop cataracts and hind leg paralysis (30).

Microscopic examination of the skeletal muscle in the present studies indicates that GSH depletion is associated with mitochondrial degeneration. The skeletal muscle abnormalities are apparently reversed on discontinuation of treatment with BSO. Skeletal muscle degeneration associated with administration of BSO can be prevented by simultaneous administration of GSH monoisopropyl ester, but not of GSH plus isopropanol. Previous studies showed that mono(glycyl) esters of GSH, in contrast to GSH itself, are effectively transported into many tissues (including heart; skeletal muscle was not studied) and converted to GSH intracellularly (7, 31, 32). In the present studies, administration of GSH monoisopropyl ester prevented the decrease in GSH levels

due to BSO in skeletal muscle mitochondria; significantly higher levels of GSH as compared to control levels were found in heart mitochondria (Table 2). The findings suggest that skeletal muscle and heart mitochondria have an efficient transport mechanism for uptake of GSH from the cytoplasm. It is also possible that the ester is taken up by and hydrolyzed within the mitochondria.

It is generally believed that a major physiological function of GSH is to provide cells with a reducing environment and to destroy the reactive oxygen compounds and free radicals formed in metabolism. Heart and skeletal muscle have low levels of catalase and superoxide dismutase as compared to other tissues and therefore might be expected to be dependent on GSH-dependent reactions for detoxication of reactive oxygen species (33). A number of studies (see, for example, refs. 1, 2, 34, and 35) have shown that decreased GSH levels sensitize cells to oxidative effects produced by administration of drugs or by radiation. The present studies show that in the absence of applied stress, very marked depletion of GSH is required before skeletal muscle mitochondrial damage occurs. As little as 8% of the control level of total tissue GSH is apparently sufficient to protect heart mitochondria. There are major differences in GSH status between heart and skeletal muscle. Thus, the GSH levels in heart and in heart mitochondria are higher than those found in skeletal muscle, as is also the tissue level of γ-glutamylcysteine synthetase. GSH turns over more rapidly in heart and the return of GSH levels to normal values after depletion occurs more rapidly. The findings also indicate that GSH monoisopropyl ester is more effectively taken up by heart than by skeletal muscle. The high GSH levels found in heart mitochondria after administration of GSH monoester suggest that this compound may be a useful agent for protection of the heart against toxicity.

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