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BUTHIONINE SULFOXIMINE-INDUCED GLUTATHIONE DEPLETION

ITS EFFECT ON ANTIOXIDANTS, LIPID PEROXIDATION AND CALCIUM HOMEOSTASIS IN THE LUNG

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Abstract—The administration of buthionine sulfoximine (BSO), an irreversible inhibitor of γ-glutamylcysteine synthetase, produces glutathione (GSH) depletion in tumors, making them sensitive to drugs and radiation. During the process, it also depletes GSH from normal tissues. Certain tumors require frequent doses of BSO for several days to produce GSH depletion. In this study, we determined that this chronic GSH-deficient condition lowers the antioxidant defense of the lung by diminishing the activities of superoxide dismutase, catalase, and glutathione peroxidase and the levels of ascorbic acid and α-tocopherol. Impaired antioxidant defense leads to enhanced lipid peroxidation, as indicated by increased levels of thiobarbituric acid reactive substances and conjugated dienes. The alteration of protein thiols by lipid peroxidation, is responsible for altered Ca^{2+} homeostasis, which, in turn, leads to cell injury. Cell injury was confirmed by elevated activities of angiotensin converting enzyme and lactate dehydrogenase, increased levels of protein and lactate, and histopathological changes.

Key words: buthionine sulfoximine; glutathione; antioxidants; free radicals; lipid peroxidation; Ca^{2+} homeostasis

BSO† is an irreversible inhibitor of γ-glutamylcysteine synthetase, which is involved in the first step of GSH biosynthesis [1]. It is used to produce GSH depletion in tumors so as to make them sensitive to drugs and radiation [2]. The potential usefulness of BSO in the sensitization of cells to radiation was first demonstrated in studies on three human lymphoid cell lines [3]. Toxicity of phenylalanine mustard toward resistant and sensitive mouse leukemia cells appeared to be correlated with the cellular level of GSH. Treatment of the resistant cells with BSO led to resensitization of the tumor cells to phenylalanine mustard [4,5]. In mice bearing resistant tumors, sensitization was achieved by continuous intraperitoneal infusion of BSO, and this led to an increase in the life span of the treated animals [6]. The administration of such an inhibitor might well be expected to decrease the GSH levels of normal tissues as well as those of tumors. Subcutaneous injections of BSO significantly depleted the GSH content in tumors and normal organs [7, 8]. Hence, studies were undertaken to evaluate the toxic effects of BSO-induced GSH depletion in normal tissues. These studies concluded that there is no apparent toxicity associated with GSH depletion [9]. However, the tissue levels of GSH obtained in these experiments were neither markedly decreased nor were they maintained at low levels for long periods. Hence, it is of interest to evaluate the toxic effects of chronic GSH deficiency produced by prolonged BSO treatment. Since BSO is cleared rapidly from the system, frequent and larger doses are required. A few studies have been conducted using this protocol, and they reported that chronic GSH deficiency causes structural damage in lung, skeletal muscles, lens epithelia of newborns and epithelia of the jejunum and colon [10–13]. However, biochemical alterations that might precede the structural damage during BSO-induced chronic GSH depletion were not dealt with in detail. Hence, in the present study, attention has been focused on the antioxidant defense, lipid peroxidation, thiol status, Ca^{2+} homeostasis and resultant cell damage in the GSH-depleted condition.

MATERIALS AND METHODS

L-Buthionine-(*S*,*R*)-sulfoximine was purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. Male Wistar albino rats, weighing approximately 100 g, were divided into two groups. One group served as the experimental and the other as the control. Animals in the test group were injected i.p. with 4 mmol BSO/kg body weight twice a day for 30 days. The control animals were treated simultaneously with physiological saline. At the end of the experimental period, the animals were killed by cervical dislocation after an overnight fast. The lungs were quickly dissected out with trachea and heart enbloc. The lung was lavaged five times using 5 mL of sterile ice-cold physiological saline and

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† Abbreviations: BSO, L-buthionine-(*S*,*R*)-sulfoximine; GSH, glutathione; TBARS, thiobarbituric acid reactive substances; ACE, angiotensin-converting enzyme; and LDH, lactate dehydrogenase.

Table 1. Effect of buthionine sulfoximine-induced glutathione depletion on γ-glutamylcysteine synthetase, antioxidants and lipid peroxidation

	Control	Experimental
γ-Glutamylcysteine synthetase (μg P_i liberated/mg protein)	614.09 ± 72.75	90.65 ± 13.74*
Reduced glutathione (μg/mg protein)	7.58 ± 0.97	4.82 ± 0.78*
Ascorbic acid (μg/mg protein)	6.53 ± 0.65	5.19 ± 0.29*
α-Tocopherol (μg/mg protein)	8.75 ± 0.87	6.64 ± 0.75*
Superoxide dismutase (U/mg protein)	4.16 ± 0.46	2.48 ± 0.13*
Catalase (μg H_2O_2 consumed/min/mg protein)	61.83 ± 5.67	7.50 ± 0.55*
Glutathione peroxidase (μg GSH consumed/min/mg protein)	35.49 ± 7.29	14.69 ± 3.68*
Thiobarbituric acid reactive substances (pmol/mg protein)	770.12 ± 20.21	1150.75 ± 30.20*
Conjugated dienes (A_{233}/A_{215})	0.56 ± 0.03	0.70 ± 0.04*

Values are means ± SD for 6 animals in each group.
* $P < 0.001$.

stored in ice for further analysis. Heart and trachea were separated from alveolar tissue. The lung was homogenized using 0.1 M Tris–HCl buffer, pH 7.0, and filtered through cheesecloth. The filtrate was subjected to low-speed centrifugation to remove cell debris and nuclear fractions. The supernatant obtained was analysed for the following parameters.

To ascertain the extent of inhibition of γ-glutamylcysteine synthetase, the activity of the enzyme was analysed according to the method of Mooz and Meister [14]. The level of glutathione was determined by the method of Moron *et al.* [15]. The levels of ascorbic acid [16] and α-tocopherol [17] and the activities of the antioxidant enzymes superoxide dismutase [18], catalase [19] and glutathione peroxidase [20] were measured. For the determination of glutathione peroxidase activity a 4 mM concentration of glutathione was added to the reaction mixture as co-substrate. TBARS were analysed by the method of Utley *et al.* [21]. Conjugated dienes were also measured [22]. Total and non-protein thiols were determined [23], and the level of protein thiols was deduced.

The calcium level in microsomes, mitochondria and cytosol, which were isolated by differential centrifugation, was measured using Integrated Coupled Plasma (ICP) analysis after perchloric-nitric acid digestion [24].

The lung lavage was analysed for the activities of ACE [25], LDH [26], protein [27] and lactate [28] to assess the damage to the lungs.

Lung tissue was isolated from at least three animals at the end of the experimental period for pathological evaluation. A portion of the lung sample was fixed in formal saline, embedded in paraffin, sectioned at 6 μm, stained with hematoxylin and eosin, and observed under a light microscope.

Statistical evaluation was carried out using Student's *t*-test.

RESULTS

The administration of BSO significantly inhibited the activity of γ-glutamylcysteine synthetase ($P < 0.001$). This decrease in activity was reflected in the level of reduced glutathione, which also was found to be lowered significantly ($P < 0.001$), as shown in Table 1. Ascorbic acid and α-tocopherol contents and the activities of superoxide dismutase, catalase and glutathione peroxidase were also decreased significantly ($P < 0.001$). A significant elevation ($P < 0.001$) was observed in the levels of TBARS and conjugated dienes in the GSH-depleted condition.

Table 2. Effect of buthionine sulfoximine-induced glutathione depletion on thiol status

	Control	Experimental
Total thiols (μg SH/mg protein)	20.10 ± 3.05	15.80 ± 1.22*
Non-protein thiols (μg SH/mg protein)	9.27 ± 0.12	6.89 ± 0.47*
Protein thiols (μg SH/mg protein)	12.74 ± 1.75	8.93 ± 1.95*

Values are means ± SD for 6 animals in each group.
* $P < 0.001$.

The levels of total thiols, non-protein thiols and protein thiols were decreased significantly ($P < 0.001$) during BSO-induced GSH depletion (Table 2).

The calcium content in mitochondria ($P < 0.01$), microsomes ($P < 0.01$) and cytosol ($P < 0.05$) was decreased in the experimental group when compared with that of controls, as shown in Table 3.

The biochemical markers of lung damage are presented in Table 4. The activities of ACE and LDH were enhanced significantly ($P < 0.001$). Protein and lactate levels were also increased ($P < 0.001$) in the lavage obtained from GSH-depleted lung.

Light microscopic examination of the control lung

Table 3. Effect of buthionine sulfoximine-induced glutathione depletion on calcium homeostasis

Calcium content (ppm) in:	Control	Experimental
Cytosol	5.80 ± 0.56	4.37 ± 0.41*
Mitochondria	6.70 ± 0.26	5.19 ± 0.52†
Microsomes	10.01 ± 0.31	7.33 ± 1.15†

Values are means ± SD for 3 determinations in each group.
* $P < 0.05$.
† $P < 0.01$.

Table 4. Biochemical parameters in bronchoalveolar lavage

	Control	Experimental
Angiotensin-converting enzyme (μg hippurate released/min/mL bronchoalveolar lavage)	6.53 ± 0.18	9.45 ± 0.28†
Lactate dehydrogenase (mU/mL)	36.54 ± 1.50	55.61 ± 1.82†
Protein (μg/mL)	1214.00 ± 60.00	1568.25 ± 30.13†
Lactate (μg/mL)	368.07 ± 23.07	437.58 ± 11.93†

Values are means ± SD for 6 determinations in each group.
† $P < 0.001$.

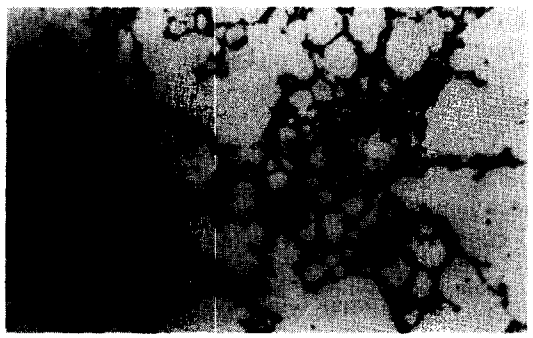

Fig. 1. Light microscopic examination of control lung showing normal bronchiole with alveolar spaces. Hematoxylin and eosin stained, 10 × 10.

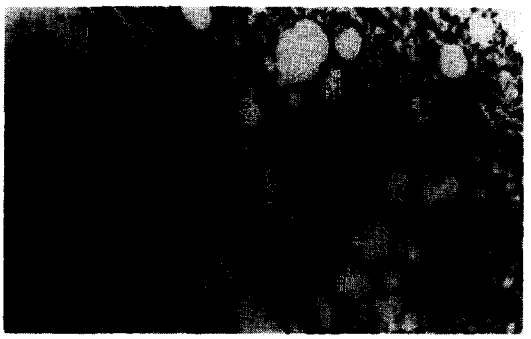

Fig. 2. Glutathione-depleted lung showing marked congestion, hemorrhage of alveolar spaces (⇒) and mononuclear cell infiltration (→). Hematoxylin and eosin stained, 10 × 10.

section (Fig. 1) showed normal bronchiole with surrounding alveolar spaces, whereas lung isolated from BSO-treated rats revealed the bronchi with marked congestion, hemorrhage of alveolar spaces, and dense mononuclear cell infiltration (Fig. 2).

DISCUSSION

Prolonged administration of BSO produced extensive loss of GSH from the lung (Table 1). Repeated and relatively high doses of BSO are required to maintain effective cellular levels of BSO and to inhibit the enzyme γ-glutamylcysteine synthetase effectively [9]. GSH, the ubiquitous peptide thiol that provides cells with their reducing environment, is a key component of the antioxidant system that defends cells against the toxic effect of oxygen [29–32]. The antioxidant defense includes the activities of such enzymes as superoxide dismutase, catalase and glutathione peroxidase and also smaller molecules such as ascorbate, α-tocopherol, GSH, β-carotene and uric acid. Glutathione peroxidase activity was found to be decreased significantly after BSO treatment (Table 1). This may be due to oxidative stress. Kerr *et al.* [33] have suggested that paraquat, which redox-cycles and produces oxidative stress, inactivates glutathione peroxidase. Diminished GSH concentration affects the regulation of reductive processes by the GSH redox pathway [34]. GSH may participate in detoxification of both superoxide

anion and hydrogen peroxide by non-specific hydrogen ion donation [35]. Although the significance of these mechanisms *in vivo* is not clear, the relative loss of this non-specific antioxidant process and decreased activity of glutathione peroxidase could increase substrate availability for both superoxide dismutase and catalase. The activities of both superoxide dismutase and catalase were decreased in the GSH-depleted lung (Table 1). This may be due to the accumulation of hydrogen peroxide. An excess of hydrogen peroxide is toxic to both superoxide dismutase [36] and catalase [37] activities. Ascorbic acid, another antioxidant, directly scavenges superoxide anion and is oxidized to dehydroascorbic acid. For the reconversion of dehydroascorbate to ascorbate, GSH is required [38]. GSH is also involved in the maintenance of α-tocopherol in the reduced state [39]. In the GSH-depleted condition, the amount of available GSH for the reconversion is low, hence, the observed decline in the levels of ascorbic acid and α-tocopherol (Table 1). Even though BSO produced an 85% inhibition of γ-glutamylcysteine synthetase, there was only a 36% decrease in the level of GSH. This may be a recovery level and appears to be consistent with that reported previously for lung [11]. This led to a corresponding decrease in the level of ascorbic acid (21%) and α-tocopherol (25%), as discussed above. Thus, GSH depletion induces an imbalance in the antioxidant defense, paving the way for oxidative stress, leading to lipid peroxidation. This is supported by an observed increase in the level of TBARS and conjugated dienes in the BSO-induced GSH-depleted condition (Table 1). Thiols are more sensitive to increased lipid peroxidation [40]. Protein thiols are essential for a number of membrane functions, such as enzyme activities and the transport system. It has been shown that some protein thiols are essential for Ca^{2+} transport in mitochondria and microsomes and that loss of such protein thiols can lead to perturbations of cellular Ca^{2+} homeostasis [41]. A strict correlation exists between lipid peroxidation and inactivation of microsomal and mitochondrial Ca^{2+} pumps [40]. Sulfhydryl groups are also involved in Ca^{2+} retention [42], and their oxidation causes the depression of Ca^{2+} sequestration in liver microsomes and mitochondria. The loss in Ca^{2+} retention appears to be related to alterations in the status of GSH and reducing equivalents [43–46], which, in turn, affects the permeability mechanism. This could be a possible reason for the observed low level of Ca^{2+} in cytosol, microsomes and mitochondria, but confirmation requires further study. Interference of Ca^{2+} homeostasis is responsible for chemically induced cellular injury. The loss in cell viability is associated with reduction in total intracellular Ca^{2+} rather than with increased intracellular Ca^{2+} [47]. The above observations evidently support the possibility of cell injury during BSO-induced GSH depletion.

Cell injury was confirmed by the increased activities of ACE, LDH and elevated levels of protein and lactate in the bronchoalveolar lavage obtained from the GSH-depleted lung (Table 4). A significant increase in ACE activity is an indication of endothelial cell damage. Lung ACE, being a constituent of the vascular endothelial membrane, is subject to perturbation by any factor that affects the integrity of the cells [48]. Increased activity of LDH indicates cell lysis. The increase in protein and lactate contents is indicative of increased alveolar capillary permeability and cell metabolism, respectively [49]. The above biochemical observations were supported by histopathological evidence, which showed marked congestion, hemorrhage of alveolar spaces, and mononuclear cell infiltration in the GSH-depleted lung (Fig. 2).

Since there is no evidence that the sulfoximine moiety itself exerts toxicity, the observed effects may be ascribed to GSH deficiency [50]. Administration of GSH monoester eliminated the BSO-induced GSH deficiency and prevented cellular damage [10], which further supports the above hypothesis.

Thus, prolonged treatment with BSO produced chronic GSH deficiency, which certainly proved to be toxic to the lung. Since the lung is the organ that is always exposed to the highest partial pressure of oxygen, the role of GSH is important. These aspects should be taken into consideration before devising any clinical trials that involve prolonged BSO treatment.

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