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COMMENTARY

ENHANCEMENT OF TISSUE GLUTATHIONE FOR ANTIOXIDANT AND IMMUNE FUNCTIONS IN MALNUTRITION

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Glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH†), a cysteine-containing tripeptide and the most abundant non-protein thiol in mammalian cells, is receiving considerable research attention (over 1200 citations in the Medline Database for 1992). The structural uniqueness of GSH, conferred by the γ-glutamyl bond, contributes to its intracellular stability (resistance to intracellular peptidases) and determines tissue specificity for uptake of extracellular GSH via γ-glutamyl transpeptidase. Tissue concentrations of GSH, like many other metabolically important compounds, are highly regulated. For example, it is difficult to deplete hepatic GSH to less than 30% of control values even with xenobiotic challenge or prolonged starvation [1–6]. Also, it is difficult to exceed the physiological maximum concentration for hepatic GSH with supplementation of GSH precursors unless hepatic GSH stores have been depleted previously with xenobiotics or by fasting [4, 6].

GSH, a substrate for GSH-*S*-transferase (EC 2.5.1.18) and GSH peroxidase (EC 1.11.1.9), was initially studied for its role in detoxification of xenobiotics and antioxidation of reactive oxygen species and free radicals. This also led to an appreciation of GSH for transport and storage of cysteine, and for the effects of nutritional status (e.g. sulfur amino acid deficiency) and physiological state on tissue GSH concentrations. With increasing knowledge, the recognized functions of GSH have been expanded to include many aspects of cell biology, such as regulation of cellular redox balance, leukotriene and prostaglandin metabolism, deoxyribonucleotide synthesis, immune function and cell proliferation [for recent GSH reviews, see Refs. 7–11]. At the same time, there has been increased interest in the potential therapeutic use of GSH or GSH precursors for treatment of toxicity or diseases, especially those conditions that are believed to be free radical-mediated and that have depleted stores of tissue GSH.

The concept of using GSH or GSH precursors therapeutically has developed from two experimental approaches. It has been demonstrated that (1) decreased tissue GSH is associated with increased toxicity and disease, and (2) supplementation of GSH precursors concomitantly with exposure to an oxidative challenge will reduce the toxicity or disease. For example, studies with experimental animal models have demonstrated that tissue GSH can be depleted by administration of compounds that oxidize or conjugate the thiol group of GSH, or of compounds that inhibit GSH synthesis [11]. Depletion of tissue GSH can also be accomplished by limiting the substrate for GSH synthesis, as in dietary sulfur amino acid deficiency [6, 12]. Decreased hepatic GSH is associated with increased toxicity to xenobiotics, such as chloroform and acetaminophen [13, 14]. Supplementation of cysteine prodrugs at the same time as administration of acetaminophen or bromobenzene protects against hepatotoxicity [4, 6, 14]. Alcoholics have an increased susceptibility to acetaminophen toxicity [15]. Treatment of acetaminophen overdose by the administration of *N*-acetylcysteine can prevent fatalities and decrease the amount of chronic liver damage in surviving patients [16].

Based on these types of studies, it has been proposed that GSH or GSH precursors could be used therapeutically for the treatment of human diseases, especially those believed to be free radical-mediated and which have depleted tissue GSH stores. In addition to those known inherited diseases due to genetic defects in GSH metabolism [17], decreased tissue GSH concentrations have been reported in several diseases. For example, GSH is decreased in the liver of patients with alcoholic liver disease [18, 19] or symptomatic Wilson's disease [20], in the substantia nigra (brain) of patients with Parkinson's disease [21], and in lung epithelial lining fluid of patients with adult respiratory distress syndrome [22] or idiopathic pulmonary fibrosis [23]. Considerable attention has been focused on the role and implications of decreased GSH status in individuals seropositive for the human immunodeficiency virus (HIV) and patients with acquired immunodeficiency syndrome (AIDS). In HIV-seropositive individuals, decreased plasma cysteine concentrations and decreased GSH concentrations in peripheral blood mononuclear cells, monocytes,

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† Abbreviations: GSH, glutathione, reduced form; PEM, protein-energy malnutrition; and OTC, L-2-oxothiazolidine-4-carboxylate.

CD4 and CD8 T-lymphocytes, plasma and lung epithelial lining fluid [24–27] may increase the risk for opportunistic infections by depressing immune function and accelerate disease progression by potentiating HIV replication [28]. The potential importance of GSH for host defense and detoxification in the lung is suggested by the relatively high susceptibility of AIDS patients to lung infections [29], particularly in the latter stages of the disease when malnutrition and wasting are common complicating factors.

However, the success of any therapeutic approach and clinical applications to various diseases will be limited by our understanding of various aspects of GSH metabolism including the synthesis, degradation, interorgan transport, cellular uptake, compartmentalization, functions and hormonal regulation of GSH. Many of these aspects of GSH metabolism, including its application in research and therapy, have been reviewed extensively by researchers with different perspectives and areas of expertise [7–11, 30–32]. The focus of this commentary is to offer a nutritional perspective on the regulation of GSH homeostasis, and to discuss potential strategies for increasing tissue GSH for antioxidant and immune functions in malnourished patients exposed to oxidative stress.

GSH in the vicious cycle of disease, infection and malnutrition

Malnutrition is a common contributing factor to the morbidity and mortality in many diseases. The classic example of wasting malnutrition that affects millions of children in developing countries is protein-energy malnutrition (PEM), also known as kwashiorkor. In affluent countries, a large number and variety of patients suffer PEM secondary to AIDS, cancer, alcoholism, chronic digestive diseases, and burns [33–37]. Decreased tissue GSH concentrations have been reported in many of these patient groups [18, 19, 24–27, 38, 39]. Decreased GSH status will contribute to a weakened antioxidant defense system and decreased immune response in malnourished individuals. Individuals with PEM are more susceptible to opportunistic infections [40]. An acute infection can precipitate the onset of a more severe stage of disease and contribute to further malnutrition and wasting [41–43]. For example, HIV-infected individuals can have relatively stable body weight and body cell mass for long periods of time, but the rapid wasting and anorexia observed during repeated secondary infections contribute to the development of AIDS [43, 44]. Prevention or successful treatment of secondary infection intervenes in this cycle of malnutrition, infection and disease [43, 45, 46].

In addition, the treatment of many diseases, even if not free radical-mediated, requires oxygen and drug therapies, both of which can increase oxidative stress. The lung is often a target of opportunistic infections in malnourished individuals [29, 47], and respiratory distress may necessitate the use of supplemental oxygen (hyperoxia), which will further increase the production of oxygen free radicals. Thus, several factors, including the disease itself, concomitant malnutrition, or oxygen and drug therapies may contribute to decreased tissue GSH concentrations in various disease states. As a result, several strategies to increase tissue GSH concentrations have been attempted, but a critical examination indicates that many of these approaches have limitations for increasing tissue GSH concentrations *in vivo* in chronic disease states. One obvious but often neglected factor is the role of various nutritional states in the regulation of GSH homeostasis in different tissues. Understanding the role of nutritional factors may contribute to the development of strategies for enhancement of tissue GSH.

Regulation of tissue GSH in adequate nutrition and malnutrition

The availability of substrate, specifically the sulfur amino acid content of the diet, is a major determinant of hepatic GSH concentration within the physiological range. In the initial rate-limiting step for GSH synthesis, cysteine and glutamate are the substrates for γ-glutamylcysteine synthetase [30]. Because plasma cysteine concentrations are relatively low, the cysteine for this reaction can also be supplied by cleavage of the disulfide cystine and by synthesis from methionine via the cystathionine pathway [30]. In the second step, GSH synthetase catalyses the reaction between glycine and γ-glutamylcysteine to form GSH [30]. In rats fed diets deficient in sulfur amino acids or low protein diets or during fasting, the hepatic GSH concentration is low; the hepatic GSH concentration is increased when low protein diets are supplemented with sulfur amino acid or with refeeding [1–3, 5, 12]. This rise and fall in hepatic GSH concentration are strictly responses to the availability of substrate, especially cysteine, in the diet for GSH synthesis [1–3, 5, 48]. Usually, this response is within a tightly regulated physiological range of hepatic GSH concentration. For example, when rats are fasted for 24 hr or fed a diet containing almost no protein (0.5%) for 2 weeks, hepatic GSH concentration does not fall below 3 μmol/g of tissue [49]. When rats are fed high protein (30 or 45%) diets with a sulfur amino acid content that is 2- to 3-fold above the normal protein (15%) diet, hepatic GSH concentration does not reach beyond the normal physiological maximum of 8–10 μmol/g [12]. The implications for human studies are that supplementation of GSH precursors may help malnourished individuals, but if hepatic GSH concentration is at the upper physiological range, then supplementation with GSH precursors will not increase the hepatic GSH concentration further, and the catabolism of excess sulfur amino acid will be an added stress for the kidney.

The locational specificity of γ-glutamyl transpeptidase determines the tissue specificity of GSH uptake and extrahepatic GSH concentration [50]. The γ-glutamyl bond of GSH can be cleaved by γ-glutamyl transpeptidase, an enzyme located on the external surface of cell membranes of various tissues. Hepatic uptake of plasma GSH is very low due to the relative absence of γ-glutamyl transpeptidase activity in the liver (Table 1) [51, 52]. On the other hand, extrahepatic tissues, such as the lung and kidney, have γ-glutamyl transpeptidase for extra-

Table 1. γ-Glutamyl transpeptidase activity in selected mouse tissues*

Tissue	γ-Glutamyl transpeptidase activity (nmol/hr/mg protein)
Kidney	26,300 ± 3,700*
Liver	3.8 ± 0.4†
Lung	25.0 ± 0.3*
Brain	71 ± 9†
Lymphocytes	41.2 ± 1.2*

* Values are means ± SD, N = 5 (reported previously in Ref. 52).

† Values are means ± SEM, N = 5 (reported previously in Ref. 51).

cellular degradation of GSH to cysteinyl glycine and γ-glutamyl amino acids. After uptake into the cell, these dipeptides are further metabolized to the amino acid constituents of GSH, and are available for intracellular GSH or protein synthesis. Export of GSH from the liver into plasma and bile is by carrier-mediated transport processes [8]. Many factors have been shown to influence the efflux process. For instance, experimental evidence indicates that efflux of hepatic GSH is enhanced by chronic exposure to alcohol [53], and by hormones and vasoactive substances that are produced under conditions of stress [54]. On the other hand, intracellular conjugated and unconjugated bilirubin and extracellular methionine appear to inhibit the export of GSH [55, 56]. The specific effects of nutritional status, especially PEM, on the efflux mechanism are still under investigation. However, Adachi *et al.* [57] reported that the calculated efflux rate of hepatic GSH in mice fed a low protein diet was significantly lower than the control group. Plasma is a mobile pool of GSH which reflects the export of GSH, mainly from the liver [58], and the uptake of plasma GSH via γ-glutamyl transpeptidase by extrahepatic tissues, including erythrocytes. Based on our observations, the blood GSH concentration, however, reflects long-term nutritional status. Blood GSH was consistently lower in PEM rats than in rats fed an adequate protein diet [49]. In other studies, long-term treatment and recovery were required to observe increases in erythrocyte GSH of children with kwashiorkor [59] or in plasma GSH of patients with acute viral hepatitis or alcoholic liver disease [60]. It has also been reported that a single oral dose of cysteine prodrugs does not increase the plasma GSH of individuals with normal plasma GSH concentrations or HIV-infected patients with decreased plasma GSH concentrations; however, increases were observed in intracellular GSH in peripheral blood mononuclear cells [27, 61]. Thus, interpretation of plasma GSH data deserves careful consideration.

In addition to the uptake of GSH via the γ-glutamyl transpeptidase mechanism, the amino acid transport mechanisms for the substrates of GSH synthesis, such as cysteine, cystine, methionine, and glutamate, may also affect the extrahepatic tissue concentrations of GSH. A diet that can cause an imbalance of the plasma amino acid profile may influence the uptake of amino acids that compete for the same transport systems [62]. This hypothesis is supported by observations that cystine uptake into cultured endothelial cells is inhibited competitively by glutamate and that the GSH concentration in these cells decreases when they are cultured in a glutamate-enriched medium [63]. Elevated plasma glutamate (up to 6-fold the normal concentration) in HIV-infected individuals [24] and in patients with advanced tumors [64] may affect extrahepatic tissue concentrations of GSH and immune responsiveness. Elevated plasma glutamate is highly correlated with decreased mitogenic responses by lymphocytes [64]. The coordinated response of macrophages and lymphocytes in T-cell-mediated immune responses is regulated, in part, by macrophage cystine uptake and subsequent cysteine release into the local environment for uptake by lymphocytes [65]. Macrophage release of cysteine is augmented by lipopolysaccharide (LPS) or tumor necrosis factor (TNF) [65] and suppressed by elevated extracellular glutamate [66]. In double-chamber experiments, it has been demonstrated that macrophages can increase the GSH concentration and DNA synthesis of mitogenically stimulated lymphocytes even when the two cell types are separated by a porous membrane [65].

The sources of GSH in the intestinal lumen include hepatic GSH exported into the bile [58], the diet, desquamated epithelial cells, and export from epithelial cells of the stomach and intestine [11]. Although direct absorption of intact GSH in vascularly perfused small intestine of the rat has been reported [67], *in vivo* studies with oral GSH have not demonstrated a sustained effect of increasing tissue GSH concentrations except in the small intestine [68–71]. The γ-glutamyl transpeptidase and dipeptidases in the intestinal tract can cleave GSH into dipeptides and free amino acids, which are absorbed and enter the circulation. Alternative forms of GSH, such as GSH ester and cysteine prodrugs that bypass intestinal digestion, are often used as dietary supplements.

Although cysteine is normally the limiting amino acid for GSH synthesis, glutamine supplementation may be beneficial for maintaining tissue GSH in situations of high energy and nutrient demand such as severe trauma. Total parenteral nutrition (TPN) formulations often contain methionine as the source of sulfur amino acid, glycine and glutamate, but they do not include glutamine. Glutamine-supplemented TPN solutions have been shown to preserve hepatic GSH and improve survival after lethal hepatic injury (acetaminophen toxicity) or following chemotherapy [72, 73]. During inflammatory stress, hepatic glutamine uptake and hepatic GSH efflux are increased when glutamine-supplemented enteral solutions are given [74]. Patients receiving glutamine-supplemented parenteral nutrition after bone marrow transplantation had improved nitrogen balance, decreased incidence of clinical infection, lower rates of microbial colonization and a shortened hospital stay compared with patients receiving standard parenteral nutrition [75]. The beneficial effects of

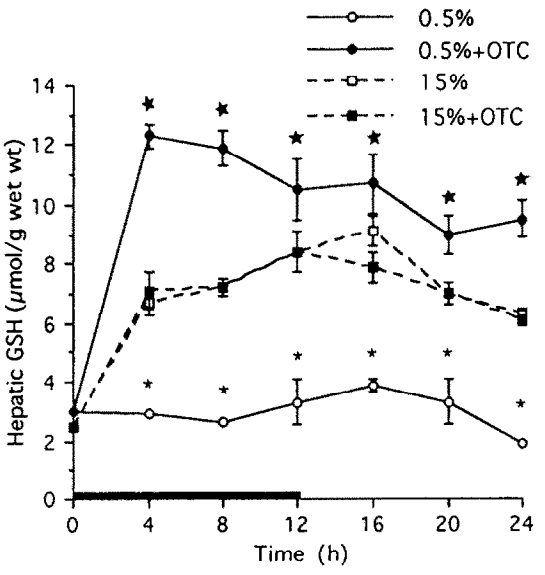

Fig. 1. Effect of oral supplementation of OTC on hepatic GSH concentration during a 24-hr period in rats fed 0.5% or 15% protein diets. Values are expressed as means ± SEM, N = 5 or 6. Significant ($P < 0.05$) main effects are diet, time, and diet × time interaction. Values marked with a star were significantly different from values in both the 0.5% and 15% groups. Values marked with an asterisk were significantly different from values in either the 0.5% + OTC or 15% group. Data are reprinted with permission from *FASEB J* **6**: 3101–3107, 1992. Copyright (1992) Federation of American Societies for Experimental Biology [Ref. 49].

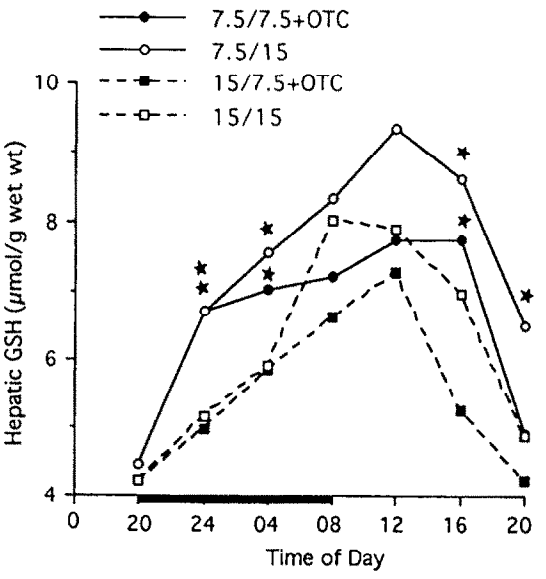

Fig. 2. Comparison of acclimatization to a diet deficient in protein (7.5%) with acclimatization to a diet adequate in protein (15%) on the 24-hr response of hepatic GSH concentrations of rats fed either a 7.5% protein diet supplemented with OTC or a 15% protein diet during the treatment period. Values marked with a star were significantly different ($P < 0.05$) for rats acclimatized to the 15% protein diet. Data are reprinted with permission from *J Nutr* **118**: 1048–1054, 1988. Copyright (1988) American Institute of Nutrition [Ref. 48].

glutamine supplementation during severe trauma may include increased substrate availability and provision of energy for GSH synthesis. In addition, glutamine synthesis and release by skeletal muscle during trauma, sepsis, surgery and burns are important for functioning of the immune system because lymphocytes and macrophages have a high rate of glutamine utilization for the production of energy [76].

The effect of nutritional status on GSH synthetic enzymes can also influence tissue concentration of GSH. The first enzyme in GSH synthesis, γ-glutamylcysteine synthetase, is known to be regulated *in vitro* by feedback inhibition of GSH [77]. Unfortunately, there is not very much *in vivo* data on the activities of GSH synthetic enzymes in humans or experimental animal models, particularly in the case of malnutrition. As previously mentioned, excess dietary protein or sulfur amino acid does not increase the maximum GSH concentration beyond the level found when a diet adequate in protein is fed [12, 48]. This supports the literature hypothesis that maximum GSH concentration is regulated by feedback inhibition of γ-glutamylcysteine synthetase by GSH. In addition, the GSH-synthesizing enzymes are reported to be maintained during starvation [1] and presumably would be maintained during protein deprivation. This was demonstrated experimentally when rats fed 0.5% protein diets (which produce severe malnutrition and wasting) were given an oral supplement of the cysteine prodrug, L-2-oxothiazolidine-4-carboxylate (OTC), and there was a rapid increase in hepatic GSH to a concentration even higher than normally found at the peak concentration of the diurnal rhythm of rats fed a normal protein diet (Fig. 1) [49]. Also, AIDS patients with the wasting syndrome were able to respond to an oral dose of *N*-acetylcysteine with increases in mononuclear cell GSH, and this would suggest that the GSH-synthetic machinery was maintained [27]. It is speculated that GSH-synthesizing enzymes are maintained even in severe malnutrition for readiness of GSH synthesis upon substrate availability, and it may be necessary for this mechanism to be in place for cell survival related to the multiple functions of GSH.

Although the amount of substrate in the diet is important to the tissue GSH, the previous dietary protein status also affects the response of hepatic GSH concentration to sulfur amino acid supplementation. For example, supplementation with OTC to rats previously fed a normal protein (15%) diet for 2 weeks did not change the rate of increase or the peak concentration of hepatic GSH of the diurnal cycle compared with the unsupplemented group (Fig. 2) [48]. However, in rats that were previously fed for 2 weeks a low protein (7.5%) diet and then supplemented with OTC, the hepatic GSH concentration increased more rapidly and was sustained at a higher concentration than in rats that

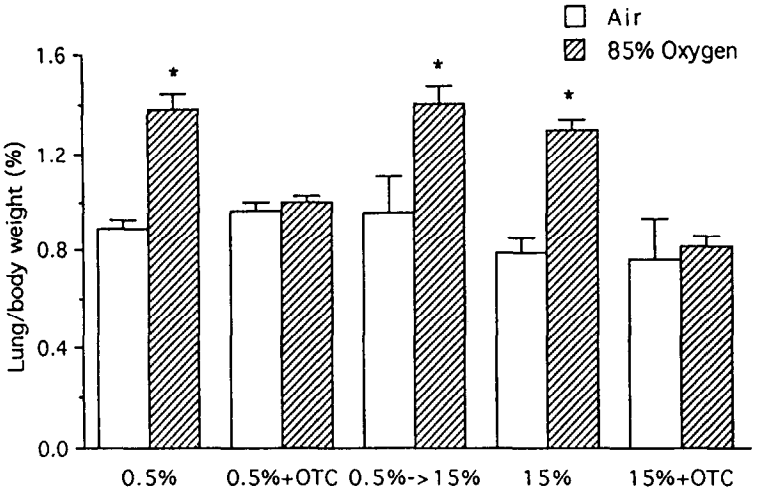

Fig. 3. Effect of hyperoxia exposure and oral supplementation of OTC or repletion with diet adequate in protein on lung/body weight ratios of rats fed 0.5% or 15% protein diet. Weanling rats were fed 0.5% or 15% protein diet for 2 weeks and exposed to 85% oxygen or air for 4 days. During the exposure period, rats fed the 0.5% or 15% protein diet were given a daily oral supplement of OTC. In rats fed the 0.5% protein diet, OTC supplementation was compared to repletion with diet adequate in protein (0.5% → 15%). Values are expressed as means ± SEM, N = 4. Significant ($P < 0.05$) main effects are diet, exposure, and diet × exposure interaction. Values marked with an asterisk were significantly different from the respective air-exposed control and the respective OTC-supplemented group. Data are adapted with permission from *FASEB J* **6**: 3101–3107, 1992. Copyright (1992) Federation of American Societies for Experimental Biology [Ref. 49].

were previously fed for 2 weeks a normal protein (15%) diet (Fig. 2) [48]. In rats, previously fed a 0.5% protein diet for 2 weeks, this initial increase was even more pronounced than in rats fed the 15% protein diet and the peak concentration exceeded the physiological maximum (Fig. 1) [49]. The difference in response to OTC between rats fed low protein diets and rats fed adequate diets is not readily explained, even though hepatic GSH concentration was similar before OTC supplementation (i.e. the feedback inhibition mechanism is not involved) and the amount of supplementation was identical for both low and normal protein groups. It has been observed in our laboratory that rehabilitation of PEM rats with a diet adequate in protein is not as effective for increasing tissue GSH concentrations for protection against pulmonary oxygen toxicity when compared with supplementation with a single substrate, the cysteine prodrug OTC, without dietary repletion of energy and other nutrients (Fig. 3). With malnourished individuals, another consideration is that immediate rehabilitation with a high-energy diet could be contraindicated. It has been shown that immediate rehabilitation with a high-energy diet containing high polyunsaturated fatty acids, iron or protein hydrolysates often results in a high mortality rate in children with kwashiorkor [78, 79]. It is speculated that malnourished patients can respond readily to the supplementation of a single substrate, such as cysteine prodrugs. A strategy to rapidly restore GSH for both antioxidant and immune defense systems during the early stabilization period along with the nutritional and therapeutic treatments may improve the survival and recovery rate of wasted patients.

Strategies to increase tissue concentrations of GSH

Various approaches have been used to increase tissue GSH concentrations [80]. Meister [11] has recently reviewed authoritatively the subject of delivery systems of cysteine and GSH for increasing cellular GSH in research and therapy. We will only comment on the effectiveness of a few popular strategies for increasing tissue GSH concentrations.

Administration of GSH by oral, intraperitoneal, intratracheal or intravenous routes does not have a sustained effect for increasing tissue GSH concentrations except in the intestinal tract with oral GSH [52, 68, 69, 71, 81–85]. Oral GSH given to animals treated with an inhibitor of GSH synthesis, buthionine sulfoximine (BSO), does not restore tissue GSH [68, 71]. GSH in the intestinal tract is subject to digestion to its constituent amino acids, which enter the circulation. Intravenous administration of GSH has not been successful due to its very short half-life [81, 82, 86, 87]. Intratracheal administration of liposome-entrapped GSH or an aerosolized solution of GSH seems to be successful for increasing GSH concentrations in the epithelial lining fluid of the lung but it is sustained for only a short time [86, 88]. When GSH was administered intraperitoneally to mice treated with BSO, an inhibitor of GSH synthesis, plasma GSH concentrations increased by 90-fold (58 μM to 5 mM), but there was no increase of GSH concentrations in tissues, i.e. liver, lung and lymphocytes [52]. This

illustrates that GSH does not freely enter cells and is dependent on the γ-glutamyl transpeptidase mechanism for intracellular uptake.

Another approach has been to modify GSH structurally to increase uptake into cells and to bypass the synthetic steps that are regulated by feedback inhibition and require ATP. Thus, GSH monoester (L-γ-glutamyl-L-cysteinylglycyl isopropyl ester) given intraperitoneally daily has been used successfully in BSO-treated mice to restore tissue GSH concentrations and prevent morphological damage to the lung, lymphocytes, intestinal tract, skeletal muscle and eye lens [52, 69, 84, 85]. In rats not previously depleted of GSH, an intravenous bolus of GSH monoester increased the GSH concentration in plasma, liver, kidney and ileal mucosa but not in the lung and spleen when measured 4 hr after administration [87]. However, the widespread therapeutic application of GSH monoesters in patients with malnutrition and developmental immaturity requires careful investigation. Metabolism of GSH monoethyl ester will release ethanol [89], and one of the detoxification pathways for ethanol is metabolism to acetaldehyde, which in high concentrations can conjugate and deplete GSH. Martensson *et al.* [85] have reported that newborn rats do not tolerate repeated doses of GSH monoester or equivalent amounts of ethanol or isopropanol as well as adult rats.

Oral supplementation of sulfur amino acids has been successful in repleting tissue GSH in experimental models of protein or sulfur amino acid deficiency or fasting [1–3, 5, 12]; however, there are some limitations for the use of sulfur amino acids. Cysteine and methionine are toxic at high doses [90], and oral or intraperitoneal administration of high doses of cysteine has been shown to decrease hepatic GSH concentrations [91]. Plasma and intracellular cysteine concentrations are relatively low when compared with other amino acids. Excess intracellular free cysteine is readily catabolized by cysteine dioxygenase (EC 1.12.11.20) [32]. It has been shown that cysteine dioxygenase activity increases dramatically with dietary protein concentrations higher than 20%, but the activity in livers of rats fed 2.1% protein is very low [92]. In addition, cysteine in solution is readily oxidized to cystine which is relatively insoluble at physiological pH, and this presents an obstacle for its addition to enteral and parenteral solutions. Methionine is generally the source of sulfur amino acid in enteral and parenteral solutions. However, the conversion of methionine to cysteine via the cystathionine pathway may be impaired in premature infants, in patients with severe liver disease, and after surgical stress [93–95].

Some of the limitations of sulfur amino acid supplementation have been overcome by cysteine prodrugs that are converted intracellularly to cysteine. *N*-Acetylcysteine is commonly used as an antidote for acetaminophen toxicity in humans [96], and its efficacy in the treatment of HIV and AIDS patients is currently being evaluated. The rate of oxidation of *N*-acetylcysteine in water or human blood plasma is considerably less than that of cysteine [91]. Considerable information is available on its pharmacokinetics with oral or intravenous administration [96–98]. Orally administrated *N*-acetylcysteine is rapidly absorbed, deacetylated and catabolized in the intestine wall and liver, resulting in approximately 10% bioavailability [97]. *N*-Acetylcysteine administered by the oral or intravenous route to patients transiently increases plasma cysteine and GSH concentrations in plasma, erythrocytes and bronchoalveolar lavage fluid [99]. At high doses, however, oral *N*-acetylcysteine administration may result in nausea, vomiting and diarrhea, and with intravenous administration, some individuals may experience anaphalatic reactions including angioedema, bronchospasm, flushing and hypotension [98].

Another cysteine prodrug, OTC, was more effective than *N*-acetylcysteine for restoring hepatic GSH in rats that had been depleted of GSH [4]. OTC is metabolized intracellularly by 5-oxo-L-prolinase to *S*-carboxy-L-cysteine, which spontaneously decarboxylates to yield L-cysteine. The attraction of using OTC for enhancing tissue GSH is that it is an intracellular cysteine delivery system for slow-release of cysteine by a naturally occurring enzyme involved in GSH synthesis, oxoprolinase (EC 3.5.2.9), which is found in almost all tissues except the erythrocyte and ocular lens [100]. Studies with [^{35}S]OTC indicate that it is transported into many tissues and that the ^{35}S-label is incorporated into GSH [80]. In humans, oral OTC administration increased the GSH concentration in lymphocytes [61]. The activity of oxoprolinase, similar to other GSH synthetic enzymes, seems to be maintained during PEM [1, 49]. The slow release of cysteine by OTC avoids the induction of cysteine dioxygenase and produces a sustained and efficient intracellular availability of cysteine for GSH synthesis. OTC supplementation, however, does not escape factors such as feedback inhibition and nutritional regulation of GSH synthesis. Thus, OTC supplementation does not always produce a dramatic increase in tissue GSH concentration when compared with GSH monoester [11]. The LD_{50} of OTC has been determined in Wistar rats and is equal to 875 mg/kg by oral dosing and 494 mg/kg by intraperitoneal administration [101].

Therapeutic effect of OTC on oxidative stress in PEM

An experimental model is often needed for demonstrating definitively the therapeutic effect of increased tissue GSH for protection against oxidative stress, especially in malnutrition. In this laboratory, we have demonstrated the effectiveness of OTC for increasing tissue GSH concentrations in PEM rats [48, 49]. The tissue response to OTC supplementation was evaluated in 24-hr time–course studies and during 4 days of hyperoxia to determine if sustained increases in tissue GSH can be achieved for protection against oxygen toxicity. Using this substrate and nutritional approach, several important principles about the regulation of tissue GSH have emerged. Taken together with results from other laboratories, these studies provide important information to be taken into consideration for the clinical application of strategies to restore tissue GSH concentration, particularly in malnourished patients exposed to oxidative stress.

Table 2. GSH concentration 24 hr after a single dose of OTC*

	GSH (μmol/g wet weight tissue)			
	15% Protein		0.5% Protein	
Tissue	−OTC	+OTC	−OTC	+OTC
Liver	8.51 ± 0.67^b	8.99 ± 0.52^b	2.21 ± 0.64^a	12.60 ± 0.85^c
Lung	3.25 ± 0.17^b	3.67 ± 0.08^b	2.06 ± 0.12^a	3.64 ± 0.14^c
Spleen	3.23 ± 0.13^b	3.22 ± 0.06^b	2.75 ± 0.28^a	4.15 ± 0.32^c

* Values are means ± SEM, N = 4 rats. For each tissue, values with different letters were significantly different ($P < 0.05$).

For our studies, hyperoxia exposure (85% oxygen) was used as a clinically relevant model of oxidative stress. Patients with respiratory insufficiency or dysfunction, for example, due to developmental immaturity of the lung, respiratory infection, or complications of other diseases, often require supplemental oxygen in order to survive. However, malnourished individuals are at greater risk for developing hyperoxia-induced organ damage. Premature infants are in a precarious nutritional state with low stores of antioxidant nutrients, and during hyperoxia exposure, they are at risk for developing chronic damage to the lungs (bronchopulmonary dysplasia) and eyes (retrolental fibroplasia) [102]. In HIV and AIDS patients, decreased GSH status, particularly in the lung epithelial lining fluid [24–27], and concomitant malnutrition or wasting syndrome [35, 42, 43] would be expected to increase the risk for developing hyperoxia-induced lung damage. In experimental animal models of protein or sulfur amino acid deficiency, decreased lung GSH concentrations contribute to the increased susceptibility to pulmonary oxygen toxicity [49, 103, 104].

During hyperoxia exposure, the protective mechanism is to increase GSH in lung tissue and bronchoalveolar lavage fluid, and antioxidant enzymes in the lung [49, 103, 105], regardless of the nutritional status. Even in PEM rats, there was a small but significant increase in lung GSH and antioxidant enzymes after 4 days of hyperoxia exposure [49; unpublished observations]. The enzymes for GSH synthesis appear to be maintained in severe malnutrition [1, 49], but during hyperoxia exposure, the lung GSH response in PEM rats is limited by availability of cysteine substrate [103]. Our strategy was to use the cysteine prodrug, OTC, for slow intracellular release of cysteine to increase substrate availability for GSH synthesis in PEM.

Oral supplementation of a single dose of OTC to PEM rats was effective for rapidly increasing liver, lung and spleen GSH concentrations, without the complete nutritional support of amino acids and energy (Table 2). We were surprised that weanling rats fed a diet containing practically no protein for 2 weeks, and which lost 20% of their initial body weight, were still able to respond rapidly to OTC supplementation and maintain the ability to synthesize GSH. Thus, it is possible to supplement wasting malnourished rats with a single substrate, OTC, to increase hepatic GSH concentrations quickly and to enhance GSH status for antioxidant and immune functions during the early stabilization phase of rehabilitation.

In the clinical setting, however, the treatment of patients who are malnourished often includes concomitant drug and oxygen therapies that expose these patients to additional oxidative stress. Thus, in our experimental animal model of PEM, it was relevant to evaluate the efficacy of OTC supplementation for providing sustained increases in tissue GSH for protection against oxidative stress. Indeed, OTC supplementation protected PEM rats against hyperoxia-induced lung damage assessed by magnetic resonance imaging (MRI) [49] and by lung/body weight ratios as an index of pulmonary oxygen toxicity (Fig. 3). Surprisingly, supplementation of PEM rats (0.5% protein) with a complete diet (15% protein) was not effective for increasing lung GSH concentrations or protecting against pulmonary oxygen toxicity, as indicated by the increase in the lung/body weight ratio (Fig. 3). It is possible that repletion of PEM rats with a complete diet, containing all the amino acids and energy, provides the stimulus and substrates for protein synthesis, of which only a small portion of sulfur amino substrate is available and used for GSH synthesis. However, supplementation of the cysteine prodrug as a single substrate maintains GSH synthesis in PEM rats, but the other amino acids become the limiting factor for synthesis of other peptides and proteins.

In addition to the benefits of OTC supplementation for protecting severely malnourished rats from pulmonary oxygen toxicity, OTC supplementation also protected rats fed diet adequate in protein (15%) from the hyperoxia-induced increases in lung/body weight ratios, which were observed in the non-supplemented 15% protein group (Fig. 3). This protective response from OTC supplementation in adequately nourished rats exposed to hyperoxia was not explained by comparing the tissue GSH concentrations of the various experimental groups. In the absence of oxidative stress, OTC supplementation to rats fed 15% protein did not increase GSH concentrations in liver, lung, kidney and blood above the physiological maximum for each tissue [49]. In the presence of hyperoxia stress, the lung GSH concentration in the OTC-supplemented 15% protein

group was not different from the hyperoxia-induced increase in lung GSH concentration, which was observed in the non-supplemented 15% protein group [49]. However, if the oxidative stress is increasing GSH turnover, then OTC supplementation to adequately nourished rats may be beneficial for increasing substrate availability for GSH synthesis and maintaining GSH concentrations. In agreement with these observations, Burgunder *et al.* [106] have reported that *N*-acetylcysteine had no effect on plasma GSH in humans in the absence of oxidative stress, but that *N*-acetylcysteine appeared to prevent depletion of plasma GSH and cysteine after the administration of a large therapeutic dose of acetaminophen. Thus, it would appear that tissue GSH concentrations do not have to be decreased to see a beneficial effect of supplementation with cysteine prodrugs in adequately nourished subjects exposed to oxidative stress.

In summary, intracellular GSH concentration plays a critical role in antioxidant and immune functions, two intimately related defense mechanisms. Special attention should be paid to the role of GSH in breaking the vicious cycle of malnutrition, infection and disease, since it affects both developing and affluent countries. Strategies to increase the tissue GSH concentration for prevention and treatment of disease need to be based on a sound understanding of the regulation of GSH homeostasis. Further experiments are required to investigate the role of GSH in body defense, and to test the metabolic and clinical efficacy of enhancing tissue GSH for antioxidant and immune functions.

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