Thiol homeostasis and supplements in physical exercise[1–4]

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ABSTRACT   Thiols are a class of organic sulfur derivatives (mercaptans) characterized by the presence of sulfhydryl residues. In biological systems, thiols have numerous functions, including a central role in coordinating the antioxidant defense network. Physical exercise may induce oxidative stress. In humans, a consistent marker of exercise-induced oxidative stress is blood glutathione oxidation. Physical training programs have specific effects on tissue glutathione metabolism that depend on the work program and the type of tissue. Experimental studies show that glutathione metabolism in several tissues sensitively responds to an exhaustive bout of exercise. Study of glutathione-deficient animals clearly indicates the central importance of having adequate tissue glutathione to protect against exercise-induced oxidative stress. Among the various thiol supplements studied, *N*-acetyl-L-cysteine and α-lipoic acid hold the most promise. These agents may have antioxidant effects at the biochemical level but are also known to influence redox-sensitive cell signaling.   *Am J Clin Nutr* 2000;72(suppl):653S–69S.

KEY WORDS   Antioxidant, *N*-acetyl-L-cysteine, glutathione, fatigue, lipoic acid, muscle, nutrition, oxidative stress, performance, redox, training

INTRODUCTION

Some 2–3 billion years ago, oxygen was introduced into the earth's atmosphere through the evolution of oxygen-releasing photosynthetic organisms. Within a few million years, the atmospheric content of oxygen rose to 21%. This shift to an environment containing oxygen provided a selective pressure for the evolution of oxygen-requiring organisms. Sixty-five percent of the adult human body is made up of oxygen (1), most of it in water. Oxidative metabolism provides an enormous advantage to aerobic organisms by allowing for the complete combustion of glucose. Correspondingly, evolution in an oxygen-rich atmosphere led to the development of endogenous antioxidant defense systems that cooperate to detoxify reactive oxygen and nitrogen species.

Thiols are ubiquitously distributed in aerobic life forms and have multifaceted functions, including a pivotal role in antioxidant defense (**Figure 1**). Added protection against oxygen toxicity is provided by exogenous antioxidants obtained primarily as nutrients or nutritional supplements (9). The present paper introduces thiols, then focuses on their significance relative to exercise-induced oxidative stress and nutritional supplements.

Thiols are not single antioxidant compounds; they are a class of organic sulfur derivatives characterized by the presence of sulfhydryl residues (-SH) at their active site. Chemically, thiols are mercaptans (C-SH), and biological mercaptans are often called biothiols. The biothiols can be classified as large molecular weight protein thiols and low molecular weight free thiols. The side chain functional CH_2-SH group of cysteinyl residues serves as an active site for most biologically important thiols. Disulfide linkages (-S-S-) between 2 -SH residues are important determinants of protein structures, such as those in insulin. In wheat, glutein subunits are cross-linked by disulfide bonds, making the wheat protein insoluble in water. Reduction of only a few essential disulfide bonds can remarkably influence dough properties. Such knowledge about the chemistry of dough has led to the use of the halogenates potassium bromate and iodate and oxidizers such as dehydroascorbate to improve bread (10).

Another characteristic feature of most thiols is their ability to act as reducing agents. Reactive oxygen species have a strong tendency to transfer electrons to other species (ie, to oxidize). Reducing agents such as thiols have negative standard reduction potentials and thus act as prompt electron acceptors (**Table 1**). Therefore, in the case of an oxidant-thiol interaction, the oxidant is neutralized to a relatively less toxic byproduct at the expense of the reducing power of thiol, which itself gets oxidized to a disulfide (C-S-S-C). A thiyl radical (C-S•) is produced when a thiol (C-SH) loses the H atom from the -SH group or loses an electron from the sulfur, followed by a proton. Under conditions of physiologic pH, thiyl radicals are unstable and may recombine to form the corresponding disulfide (12). In biological systems, there are specific reductases that recycle disulfides to thiols using cellular-reducing equivalents such as NADH or the corresponding phosphorylated form (NADPH) (Figure 1). In this way the power of cellular metabolism is coupled to maintain a favorable oxidoreductive (or redox) state of thiols.
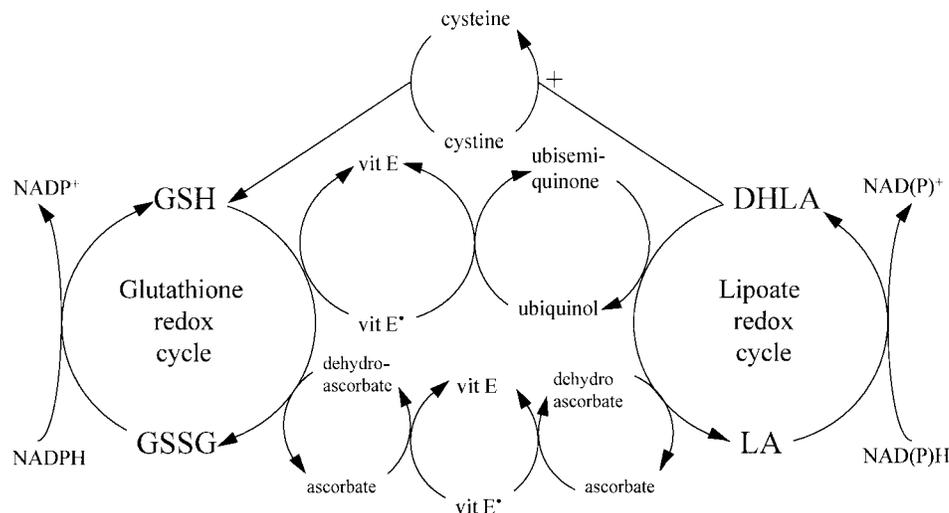
FIGURE 1. Thiol redox cycles play central roles in the antioxidant defense network. Both glutathione and lipoate redox cycles can be driven by cellular-reducing equivalents to generate their respective reduced forms (GSH and DHLA). The ability of lipoate to increase cell GSH is mediated by the reduction of cystine to the GSH precursor cysteine by dihydrolipoate. LA, lipoate; GSSG, oxidized glutathione; vit, vitamin. For more details on antioxidant interaction, *see* references 2–8.

CENTRAL ROLE OF THIOLS IN THE ANTIOXIDANT NETWORK

In biological systems, the term *oxidative stress* refers to an imbalance favoring prooxidants over antioxidants. Defense against oxidative stress depends primarily on an orchestrated synergism between several endogenous and exogenous antioxidants (Figure 1). For example, vitamin E is a major lipid-phase antioxidant that protects against oxidative lipid damage. Although vitamin E can effectively terminate lipid peroxidation chain reactions, during the course of such reactions vitamin E can itself be oxidized to a tocopheroxyl radical. Inadequate recycling of this oxidized form of vitamin E to the potent reduced form may lead to accumulation of oxidized vitamin E, which may even lead to the initiation of pathologic processes (2, 13). In biological membranes, vitamin E is present in a low molar ratio in contrast with phospholipids, which are abundant and highly susceptible to oxidative damage. For every 1000–2000 molecules of phospholipid, it is estimated that only 1 molecule of vitamin E is present for antioxidant defense. In addition, lipid peroxyl radicals, reactive oxygen species that can trigger lipid peroxidation reactions, are continuously produced in biological membranes (14). The ability of vitamin E to protect membranes under such remarkably adverse conditions can be explained by the continuous recycling of this vitamin as it acts as an antioxidant (2–8), which permits it to regain antioxidant potency. Thiols such as reduced glutathione (GSH) and dihydrolipoate support the recycling of vitamins C and E (Figure 1). In addition to their central role in antioxidant biochemistry, thiols have such functions as protein synthesis and structure, redox-sensitive signal transduction, cell growth and proliferation, regulation of programmed cell death, xenobiotic metabolism, and immune regulation (**Figure 2**).

ENDOGENOUS BIOTHIOLS

The discovery of biothiols dates back to 1888, when de Rey-Pailhade (18, 19) observed that yeast cells contain a substance that facilitates the formation of hydrogen sulfide when the cells are crushed with elemental sulfur. He found as well that this substance had many other sources, eg, fish and bovine muscle, bovine liver, fresh sheep blood, sheep brain, egg white, the small intestine of the lamb, and tips of fresh asparagus. In light of the substance's affinity for reacting with sulfur, de Rey-Pailhade named it philothion (philo = love and thion = sulfur in Greek). Later, Hopkins (20) observed that philothion in muscle, liver, and yeast could be extracted with water, and he suspected that philothion was a dipeptide containing glutamate and cysteine. In 1921 Hopkins changed the name to glutathione. Eight years later, Hopkins (21) and Kendall et al (22) independently discovered that GSH was actually a tripeptide and that the peptide contained glycine (Glu-Cys-Gly). Since the discovery of the tripeptide, GSH research has attracted considerable interest, particularly because of the apparent ubiquity of the low molecular weight thiol in all living cells (23, 24). Today, GSH is recognized as a key physiologic antioxidant that not only detoxifies reactive oxygen species directly but also enhances the functional ability of other crucial exogenous antioxidants, such as vitamins E and C (Figure 1).

The GSH-dependent detoxification of reactive oxygen species is accomplished through 2 general mechanisms: *1*) direct or spontaneous reaction with reactive oxygen species and *2*) glutathione peroxidase catalyzed decomposition of reactive oxygen species both of which produce glutathione disulfide (GSSG, also known as oxidized glutathione) (Figure 1). Intracellular GSSG, thus formed, may be reduced back to GSH by glutathione disulfide reductase (GSSG reductase) or released to the extracellular compartment. Another family of GSH-dependent enzymes, glutathione transferases, catalyzes the detoxification of electrophilic xenobiotics, rendering them more water soluble for excretion (25). Thioredoxin and glutaredoxin are other endogenous biothiols known to participate in important redox processes; both are small proteins with active center dithiols [-(SH)$_2$] in their reduced forms (26). This center, by way of rapid thiol-disulfide interchange, participates in redox reactions.

TABLE 1
Reduction potential of some biological redox couples[1]

Reduction half-reaction	Standard reduction potential
	V
$1/2\ O_2 + 2\ H^+ + 2\ e^- \rightarrow H_2O$	0.816
$Fe^{3+} + 2\ e^- \rightarrow Fe^{2+}$	0.771
Pyruvate $+ 2\ H^+ + 2\ e^- \rightarrow$ lactate	−0.185
Cystine $+ 2\ H^+ + 2\ e^- \rightarrow 2$ cysteine	−0.22
GSSG $+ 2\ H^+ + 2\ e^- \rightarrow 2$ GSH	−0.23
Lipoic acid $+ 2\ H^+ + 2\ e^- \rightarrow 2$ dihydrolipoic acid	−0.29
Lipoyl dehydrogenase (FAD) $+ 2\ H^+ + 2\ e^-$ $\rightarrow$ lipoyl dehydrogenase (FADH$_2$)	−0.34

[1] GSSG, oxidized glutathione; GSH, reduced glutathione. Adapted from reference 11.

EXERCISE-INDUCED OXIDATIVE STRESS

In 1978 Dillard et al (27) examined the relation between physical exercise and oxidative tissue damage in humans. These researchers reported that exercise of moderate intensity increased the content of pentane, a lipid peroxidation byproduct, in expired air. This short-living free radical species can be detected directly by electron paramagnetic resonance spectroscopy. Using this technique, Davies et al (28) showed for the first time in rats that exhaustive treadmill exercise may increase by 2- to 3-fold the free radical concentration of skeletal muscle and liver. The bout of exercise decreased mitochondrial respiratory control and caused sarcoendoplasmic reticulum membrane damage. The authors developed the interesting hypothesis that exercise-induced free radical damage may stimulate mitochondrial biogenesis.

Does the contraction of skeletal muscle with the associated enhanced consumption of oxygen increase the generation of reactive oxygen species? Numerous experimental studies have addressed this question with use of the contracting muscle model. For example, Diaz et al (29) observed that hydroxyl radical, a highly reactive oxidant, is generated in fatiguing contractions. In addition, Reid et al (30, 31) observed that superoxides are produced intracellularly in muscle cells and then released to the extracellular medium. Hydrogen peroxide, produced in the contracting muscle cell, readily crosses the cell membrane to reach the extracellular medium, where it contributes to the formation of hydroxyl radicals. In this way, relatively less damaging forms of reactive oxygen such as superoxides and hydrogen peroxide can be released from the cells to produce more damaging hydroxyl radicals. Barclay and Hansel (32) showed that superoxide radicals attenuate the function and enhance the rate of fatigue of contracting muscles. Other studies indicate that strenuous exercise augments oxidative stress and that exercise-induced oxidative stress may damage biological components, eg, lipids and proteins, as well as the genetic material (8, 33, 34). Various mechanisms that may contribute to exercise-induced oxidative stress were reviewed recently (35).

SKELETAL MUSCLE GLUTATHIONE

Using a hepatectomized rat model, Kretzschmar et al (36) showed that skeletal muscles play a central role in whole-body GSH homeostasis. How much GSH is in skeletal muscle may vary by muscle type; in male Sprague-Dawley rats, relative amounts were as follows: soleus > deep vastus lateralis > superficial vastus lateralis muscle (37). Compared with other skeletal muscles, soleus muscle, which has marked oxidative metabolic capacity, also appears to have a remarkably well developed antioxidant defense system. Furthermore, in addition to higher total glutathione, the soleus muscle type has been found to have markedly greater activities of GSH-dependent antioxidant enzymes such as glutathione peroxidase, GSSG reductase, and the peroxide-decomposing enzyme catalase. Laughlin et al (38) proposed that although antioxidant enzyme activities are related to skeletal muscle oxidative capacity, the effect of exercise training on muscle antioxidant enzymes cannot be predicted by measured changes in oxidative capacity. In a study these researchers conducted in rats, treadmill training increased citrate synthase activity of triceps brachii muscle, indicating an increased oxidative capacity. In both control (sedentary) and trained rats, activities of catalase, superoxide dismutase, and glutathione peroxidase were directly correlated with the percentages of oxidative fibers in the skeletal muscle samples, suggesting that such fibers have elevated enzymatic antioxidant defense status.

TRAINING AND GLUTATHIONE-DEPENDENT TISSUE ANTIOXIDANT DEFENSES

A properly selected exercise training regimen improves cardiovascular health and the gross functional capacity of the human body. Does exercise training influence our antioxidant defenses as well? Judging from several independent experimental studies, it appears that GSH content and GSH-dependent enzyme activities respond to training (39). The work of Kihlström (40) supports the hypothesis that endurance swim training enhances protection of the heart against oxidative stress. Here, the added capacity to detoxify reactive oxygen species was mainly due to elevated GSH and a more efficient NADPH-supplying system in the trained heart. The training program decreased the activity of GSSG reductase in the myocardium and increased the activity of thioredoxin reductase. An earlier investigation by Kihlström et al (41) also found that swim training decreased GSSG reductase activity in the heart. GSSG reductase requires NADPH to maintain a favorable redox status of glutathione. In the study by Kihlström et al (41), training increased the activity of the NADPH-supplying enzyme glucose-6-phosphate 1-dehydrogenase in the right ventricle. In addition, the amount of GSH was greater in the trained heart, especially in the left subepimyocardium. In a study by Ji (42) with rats, a bout of exhaustive treadmill exercise increased the activities of glutathione peroxidase and GSSG reductase in the skeletal muscle, and a tissue-specific response to training was reported. Endurance treadmill training decreased glutathione peroxidase activity in the liver but increased the activity of this enzyme in the deep vastus lateralis muscle.

Lew and Quintanilha (43) observed that for the same amount of submaximal exercise, endurance-trained rats improved their ability to maintain tissue glutathione redox status (as reflected by the ratio of GSSG to total glutathione) in comparison with their untrained counterparts. The endurance training program significantly increased the activities of glutathione peroxidase, GSSG reductase, and glucose-6-phosphate 1-dehydrogenase in the skeletal muscle and heart tissues. A favorable change of the glutathione redox cycle in response to exercise training has also been observed in the brain, particularly in the cortex and brain stem (44).
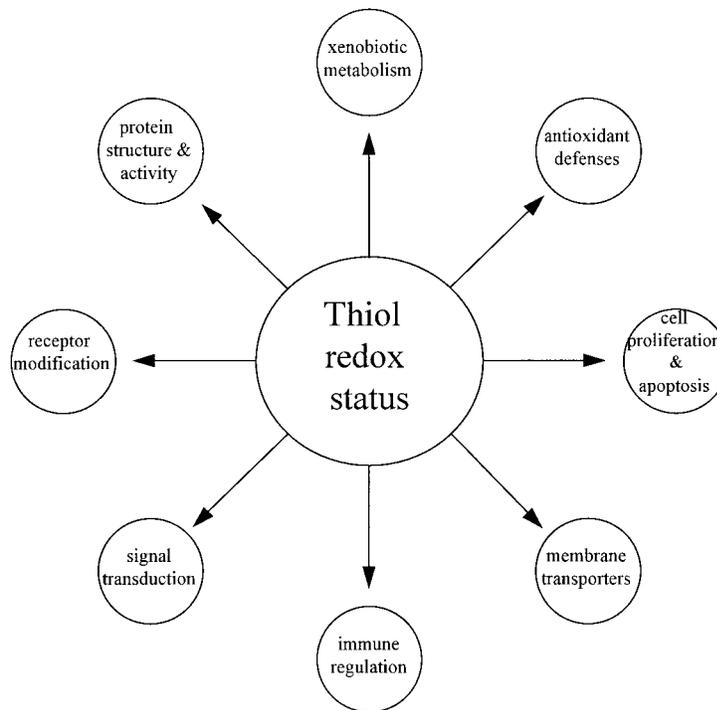
FIGURE 2. The thiol redox state plays a central role in regulating a wide variety of cell responses. *See* references 15–17.

The effects of aging and exercise training on antioxidant enzyme activities in rat skeletal muscle were tested by Ji et al (45). Superficial glycolytic and deep oxidative vastus lateralis muscles were collected from rats ranging in age from 2.5 mo (young) to 27.5 mo (senescent). Old rats had significantly lower glutathione peroxidase activity in the deep vastus lateralis muscle. After progressive treadmill training, the activity of the hydroperoxide-metabolizing enzyme in deep vastus lateralis muscle increased significantly to a point where it was greater than that observed in sedentary young rats. The authors concluded that although aging can adversely influence antioxidant enzyme capacity in skeletal muscle, regular exercise can preserve such protective function. Similarly, in an earlier study by Ji et al (46), an increase was seen in rat skeletal muscle glutathione peroxidase activity from endurance treadmill training. In a different model, Kanter et al (47) found consistently that swim training enhanced the activity of glutathione peroxidase in the blood and liver. In contrast, Tiidus and Houston (48) observed that in female rats, 6 wk of treadmill training did not significantly influence glutathione peroxidase activity in skeletal muscle, heart, and liver. In a human study, however, endurance training increased erythrocyte glutathione peroxidase activity (49).

Much of the research in our laboratory has been directed toward the study of tissue GSH metabolism in response to exercise and training. In one study (50), treadmill training of rats increased skeletal muscle citrate synthase activity, indicating enhanced oxidative capacity. In addition, total glutathione content of the liver was elevated in the trained rats. However, such an effect was not observed in any of the skeletal muscles studied, eg, red gastrocnemius, mixed vastus lateralis, and longissimus dorsi. Glutathione peroxidase activity in leg muscle was higher in trained rats than in untrained rats, however. Treadmill training decreased GSSG reductase activity in red gastrocnemius

muscle, a finding that may be related to the high intensity (2.1 km/h, 2 h/d, 5 d/wk, 8 wk) of training, which may have increased flavoprotein turnover and breakdown in the muscle. Endurance training also increased the activity of γ-glutamyltransferase in both leg muscles, the effect being more pronounced in red gastrocnemius. In the trained leg muscles, activated γ-glutamyltransferase may facilitate the import of substrates required for GSH generation (**Figure 3**). Thus, in the presence of extracellular cysteine, availability of this amino acid in the cell is increased.

Lipoate supplemented to cells is promptly taken up by the cells and reduced to dihydrolipoate by enzymes such as dihydrolipoamide dehydrogenase, GSSG reductase, or thioredoxin reductase (NADPH). Dihydrolipoate produced inside the cell is a powerful reducing agent that can even reduce protein disulfides to protein sulfhydryls. After being generated in the cell, dihydrolipoate is released to the extracellular medium, where it can reduce cystine to cysteine and thus improve cysteine availability inside the cell.

In the treadmill study, decreased γ-glutamyltransferase activity was observed in the control leg muscles after exercise (50), but this effect was not observed in the trained leg muscles, indicating that during exercise trained muscles have a more active substrate import system for GSH generation than untrained muscles. γ-Glutamyltransferase activity of the trained liver decreased ($\approx 50\%$) after the exercise bout; this response might ensure that fewer γ-glutamyl compounds are re-trapped in the liver when the active peripheral tissues have acute needs. The contention that exercise training strengthens GSH-dependent antioxidant defenses is supported by a more recent study in which swim training of rats was associated with a marked increase in the activities of glutathione peroxidase and GSSG reductase in the skeletal muscle, heart, and liver (53).
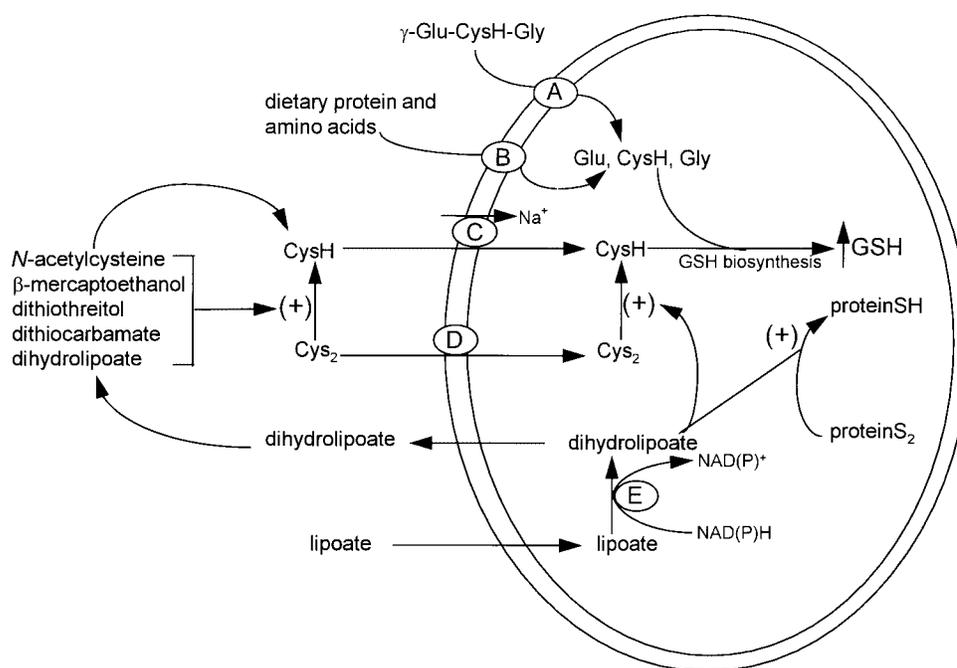
FIGURE 3. Improvement of cysteine availability inside cells is an effective strategy for enhancing amounts of intracellular glutathione. A, γ-glutamyltransferase; B, glutamine (Glu), cysteine (CysH), or glycine (Gly) transporters; C, ASC amino acid transport system (51); D, x_c^- amino acid transport system (51). A and B supply precursor amino acids for reduced glutathione (GSH) synthesis. Cystine (represented as cystine disulfide, Cys_2) is transported into the cell by D. In the presence of extracellular cysteine (CysH), however, the reduced amino acid is transported in to the cell via C. Import of cysteine via C has been estimated to be 10 times faster than cystine import via D (52). Thus, in the presence of extracellular cysteine, availability of this amino acid in the cell is increased. Lipoate supplemented to cells is promptly taken up by the cells and reduced to dihydrolipoate by enzymes such as dihydrolipoamide dehydrogenase, glutathione reductase (NADPH), or thioredoxin reductase (NADPH). Dihydrolipoate produced inside the cell is a powerful reducing agent that can even reduce protein disulfides ($proteinS_2$) to protein sulfhydryls (proteinSH). After being generated in the cell, dihydrolipoate is released to the extracellular medium, where it can reduce cystine to cysteine and thus improve cysteine availability inside the cell. Glutamate competitively inhibits cystine uptake via D, and in this way it decreases cell GSH. By reducing extracellular cystine to cysteine, which then uses C for entry into the cell, lipoate can bypass the adverse effects of excess glutamate.

GSH-dependent antioxidant protection in the skeletal muscle is influenced by the state of physical activity: endurance training enhances and restriction of chronic activity diminishes such protection (50). Beagles, commonly used as a laboratory animal, possess a well-developed musculoskeletal system apparently suited for running, and thus we studied the influence of treadmill training in these dogs. Training on the treadmill (5.5–6.8 km/h, 40 km/d, 5 d/wk, 15% uphill grade, for 40 wk) increased the oxidative capacity of red gastrocnemius, extensor carpi radialis, and triceps muscles of the leg. Training-induced changes in the components of GSH metabolism were most pronounced in the red gastrocnemius muscle, which is predominantly oxidative by composition. Total glutathione in the liver and red gastrocnemius was elevated in response to training. In all 3 leg muscles noted above, training increased glutathione peroxidase activity, and again this effect was most pronounced in the red gastrocnemius muscle. GSSG reductase activities in extensor carpi radialis and triceps muscles were higher in the trained dogs. Trained animals with higher total glutathione reserves in the liver also had higher glutathione transferase activity, indicating that the liver of the trained animals had a higher detoxicant status. Training effects were not observed in the splenius muscle of the neck and trunk region.

In a separate experiment (50), the effect of chronically restricting activity on the red gastrocnemius muscle of beagles was studied by immobilizing the knee and ankle joint of the right

pelvic limb of each dog for 11 wk in a light fiberglass cast. The left leg was used as the paired control. Chronic physical inactivity did not influence the activity of GSH-dependent enzymes, but the total amount of glutathione in the red gastrocnemius muscle was remarkably decreased in the immobilized leg. In another study, decreased total glutathione and increased GSSG were associated with atrophy of skeletal muscle (54).

In a 55-wk endurance training study with beagles, it was observed that physical training may enhance hepatic glutathione transferase activity (50). Glutathione transferases are a family of GSH-dependent enzymes that play a central role in drug detoxification and xenobiotic metabolism. In addition, glutathione transferases may contribute to hydroperoxide metabolism because they have glutathione peroxidase activity that does not depend on selenium. More recently, Veera Reddy et al (25) confirmed in a rat model that swim training results in higher hepatic glutathione transferase activity than in untrained controls. Electrophoretic and western blot analyses revealed that a Ya-sized subunit of the transferase is specifically induced by exercise training. Analyses of affinity-purified glutathione transferases further revealed that a Ya_1 subunit of Ya was most sensitive to exercise training. Untrained control rats had Ya subunits predominantly made up of Ya_2, whereas the trained animals had a 4.3-fold increase in Ya_1. Glutathione transferases of exercise-trained animals had increased peroxidase activity, an effect that was consistent with the changes

in subunit composition. Studies on the regulation of *Ya* gene expression have revealed that the gene contains a regulatory sequence known as the antioxidant response element, or ARE, in the 5′-flanking region. Transcription of Ya is activated by oxidants such as hydrogen peroxide by a mechanism acting through the ARE (55). Ya_1 is induced in hydroperoxide overload situations, such as selenium deficiency (56). Thus, exercise-induced regulation of Ya_1 may be expected to be oxidant mediated.

Powers et al (57) investigated the effect of intensity and duration of exercise on training-induced antioxidant enzyme responses; rats were exercised at low, moderate, or high intensity for 1 of 3 time periods (30, 60, or 90 min/d). The costal and crural diaphragm, plantaris muscle, and parasternal intercostal muscles were studied. Training effects were highly tissue specific. All training programs markedly increased glutathione peroxidase activity in the costal diaphragm but not in the crural diaphragm. The intensity or duration of exercise did not have a major influence on training-induced elevation of glutathione peroxidase activity in the costal diaphragm. In the crural diaphragm, however, moderate- and high-intensity exercise training decreased tissue glutathione peroxidase activity when daily exercise lasted as long as 90 min. None of the training programs influenced glutathione peroxidase activity of the parasternal intercostal muscle, but in the plantaris muscle, longer duration of daily exercise triggered a more marked response.

Results of a similar study support the idea that training effects are indeed highly tissue specific (58). In this study, although exercise training increased glutathione peroxidase activity in the red gastrocnemius muscle of rats, such effects were not seen consistently in the soleus or even white gastrocnemius muscles. As with the previous results for the plantaris muscle, the duration of daily exercise markedly affected the response in glutathione peroxidase activity. In another study, Criswell et al (59) found that high-intensity training was superior to moderate-intensity training in elevating glutathione peroxidase activity in the soleus muscle of rats.

Studies investigating the influence of physical training on tissue antioxidant status generally tested endurance training, which enhances tissue oxidative capacity. Comparable information on the effect of sprint training, which relies primarily on nonoxidative metabolism, is scanty. Atalay et al (60) examined the effect of a sprint training regimen on the rat skeletal muscle and heart GSH system. Soleus muscle, made up predominantly of slow-oxidative fibers, was studied as representative of slow-twitch muscle; plantaris and extensor digitorum longus muscles, consisting mainly of glycolytic fibers, and the superficial white portion of the quadriceps femoris muscle, consisting mainly of fast-oxidative glycolytic fibers, were studied as representative of fast-twitch muscle. Mixed gastrocnemius muscle was examined as an antagonist of extensor digitorum longus muscle. Lactate dehydrogenase and citrate synthase enzyme activities were measured in muscle to test the effects of training on glycolytic and oxidative metabolism, respectively. The efficacy and specificity of the 6-wk sprint training protocol was attested by markedly increased anaerobic but not aerobic metabolic capacity in primarily mixed and fast-twitch fiber muscles. Endurance training consistently upregulated GSH-dependent defenses and other antioxidant enzymes, with effects most marked in highly oxidative muscle (38, 50, 59, 61–63). In contrast, sprint training enhanced antioxidant defenses primarily in fast glycolytic muscle. Compared with that in the control group, glutathione peroxidase activities in gastrocnemius, extensor digitorum longus muscles, and the heart increased after sprint training. The training program also increased GSSG reductase activity in the extensor digitorum longus muscle and heart. Sprint training did not influence the amount of glutathione or GSH-related enzymes in the oxidative soleus muscle.

The effect of intermittent sprint cycle training on the degree of muscle antioxidant enzyme protection was also investigated in humans (64). Resting muscle biopsies obtained before and after 6 wk of training and 3 h, 24 h, and 72 h after the final session of an additional 1 wk of more frequent training were analyzed for activities of the antioxidant enzymes glutathione peroxidase, GSSG reductase, and superoxide dismutase. Intermittent sprint cycle training, which enhances the capacity to generate anaerobic energy, also improved the amount of antioxidant protection in the muscle. Thus, depending on the type of work program, GSH metabolism of specific tissues may be expected to respond to physical training.

EXERCISE-INDUCED CHANGES IN TISSUE GLUTATHIONE

Studies in blood

Almost all blood GSH is contained in the cellular compartment, primarily in the erythrocytes; human blood plasma contains only trace amounts of GSH. Blood glutathione homeostasis has been suggested as a determinant of resting and exercise-induced oxidative stress in young men (65). In a relatively large ($n = 265$) human study, the mean concentration of total (reduced + oxidized) blood glutathione in adults aged 18–73 y was 941 ± 155 μmol/L; that of GSH was 849 ± 163 μmol/L (66). These values were slightly lower for women, but the differences were not significant. The effect of age on the blood glutathione status of men and women is illustrated in **Figure 4**, where a trend is seen for an age-dependent increase in the ratio of blood GSSG to total glutathione. In the same study, plasma was shown to contain only 0.4% of total blood GSH. Compared with adults, children (median age: 13.3 y) had lower total glutathione, but not GSH, concentrations in blood. Individuals who practiced habitual physical activity had higher blood concentrations of both GSH and total glutathione. Smoking and alcohol consumption were associated with elevated blood GSH, perhaps as a defense against chronic oxidative stress exposure. The use of oral contraceptives tended to lower blood glutathione concentrations, but the relation was not statistically significant. Interestingly, in the population as a whole, total blood glutathione concentration was positively correlated with cholesterol and calcium concentrations (66).

Oxidation of thiols to disulfides is a sensitive marker of oxidative stress. In 1988 Gohil et al (67) were the first to report that even submaximal exercise induces blood GSH oxidation; a 100% increase in blood GSSG concentration was seen within the first 15 min of exercise at 65% of peak oxygen uptake (**Figure 5**). However, Ji et al (68), who exercised 8 healthy male cyclists at 70% of maximal oxygen uptake, found that a bout of exercise lasting >2 h did not elevate blood GSSG. Studies involving human blood GSH oxidation during exercise are limited, but previous studies showed that exhaustive exercising of rats remarkably increases GSSG concentrations in plasma (69).

Our laboratory investigated the association between exercise intensity and related oxidative stress in healthy young men who
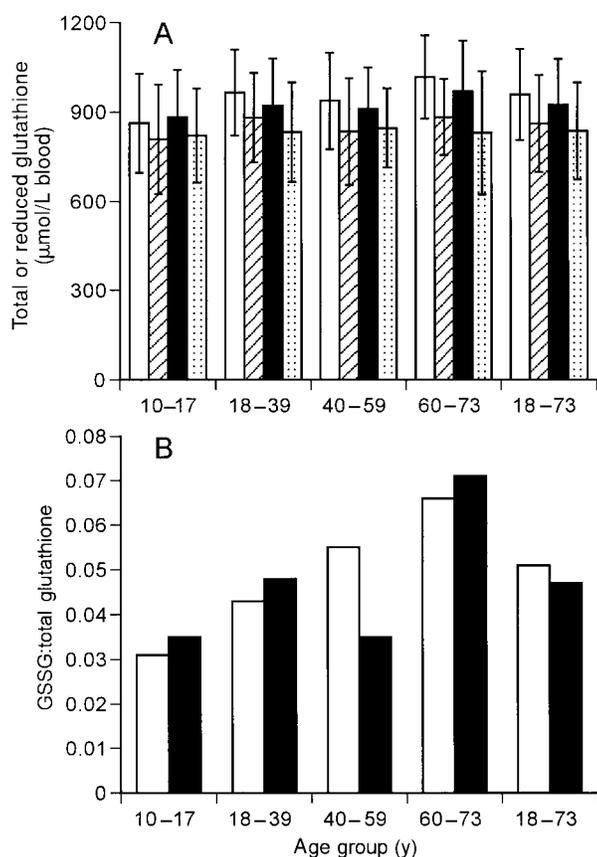
FIGURE 4. A: Mean (±SD) blood total glutathione and reduced glutathione (GSH) concentrations in men and women of different age groups as reported by Michelet et al (66). No clear sex-specific differences were observed. Open bar, total glutathione in men; hatched bar, GSH in men; filled bar, total glutathione in women; dotted bar, GSH in women. B: Ratio of oxidized glutathione (GSSG) to total glutathione in men (□) and women (■) of different age groups. GSSG data were calculated [2 GSSG = total glutathione − GSH] from results published by Michelet et al (66). A trend was seen that with increasing age, more of total glutathione in the human blood is present in the oxidized form. No sex-specific differences in blood glutathione redox state were observed.

performed 2 maximal exercise tests (mean duration: ≈14 min) and, 1 wk apart, 2 bouts of 30 min of exercise at their aerobic and anaerobic thresholds (70). Blood samples were drawn before, immediately after, and 24 h after tests. In line with the observation of Gohil et al (67), all 4 test exercise bouts notably increased the concentration of blood GSSG. Exercise-induced perturbations in blood glutathione redox status and plasma lipid peroxide concentration were no longer observed in the 24-h postexercise recovery samples. In another study, Viguie et al (71) found no evidence of persistent or cumulative effects of repeated leg cycling exercise (at 65% of peak oxygen uptake, for 90 min, for 3 consecutive days) on blood glutathione redox status. In moderately trained men, a 50% decline in the blood GSH concentration was observed during the first 15 min of exercise, which was accompanied by an increased blood concentration of GSSG. Total glutathione concentration in the blood did not change significantly during the exercise, and the blood GSH concentration returned to baseline after 15 min of recovery.

Although running at high speed (intervals of ≈20 s) to exhaustion did not influence blood GSH oxidation, Sastre et al (72) observed that in trained men, blood GSSG concentrations were 72% higher immediately after exercise than at rest. Accurately determining the ratio of GSSG to GSH is challenging primarily because GSH is unstable during sample processing. This issue was addressed by Vina et al (73), who used a modified analytic approach in showing that, compared with preexercise values, exhaustive exercise may increase blood GSSG concentrations in humans and rats by ≈1-fold and 3.5-fold, respectively. Human blood GSH oxidation has proven to be a consistent response to exercise. In young men, intermittent exercise bouts to exhaustion increased blood GSSG by 35% (49). More recently, Laaksonen et al (74) observed exercise-induced blood GSH oxidation in young men with type 1 diabetes and in corresponding healthy control subjects. Thus, from results obtained so far we may conclude that physical exercise may enhance the use of blood GSH, resulting in a decreased ratio of GSH to GSSG.

Studies in other tissues

Information on the possible effect of physical exercise on the GSH status of human tissues such as the skeletal muscles, heart, or liver is currently not available, but several studies have investigated this issue in experimental animals. Physical exercise clearly influences GSH metabolism in the skeletal muscles and liver of rats (39, 75). Lew et al (69) reported that exhaustive exercise consistently decreases both liver and muscle GSH. In investigating the influence of an exhaustive treadmill run on the tissue GSH status of rats (50), we found that the exercise decreased the total glutathione reserves of the liver and the active skeletal muscles red gastrocnemius and mixed vastus lateralis, but we did not observe this effect in the less active longissimus dorsi muscle. Exercise-induced decreases in the total glutathione pool in the liver, red gastrocnemius muscle, mixed vastus lateralis muscle, and the heart of rats were also seen in another independent study we carried out, but this effect was not seen in the lung (76). Duarte et al (77) confirmed that a single bout of exercise results in GSH loss from skeletal muscle; in their study, exercising resulted in a 50% decrease in the total glutathione content of the left soleus muscle, an effect that was interpreted as an index of oxidative stress. Recovery of the muscle GSH concentration was slow in the postexercise recovery period. This recovery was remarkably faster in mice that were supplemented with allopurinol, an inhibitor of the superoxide-producing enzyme xanthine oxidase. Hellsten (78) suggested that exercise-induced increases in superoxides generated by xanthine oxidase causes oxidative stress to muscle tissues located nearby and that this stress is manifested as a loss of tissue GSH.

Exhaustive treadmill exercise (24.1 m/min, 15% uphill grade) induces GSH oxidation in the plasma, skeletal muscle, and liver of rats (69). This effect was confirmed in our rat studies, in which exhaustive treadmill exercise markedly increased the amounts of GSSG in the liver, red gastrocnemius muscle, mixed vastus lateralis muscle, blood, and plasma (76). After oxidant challenge, GSH is transformed within the cell to GSSG. When the rate of oxidation is low, much of the GSSG thus produced may be enzymatically reduced by GSSG reductase activity to GSH. However, with a more severe oxidative stress, the rate of GSSG reduction cannot match the rate of its formation, which may result in the accumulation of intracellular GSSG. High concentrations of intracellular GSSG may be cytotoxic. In
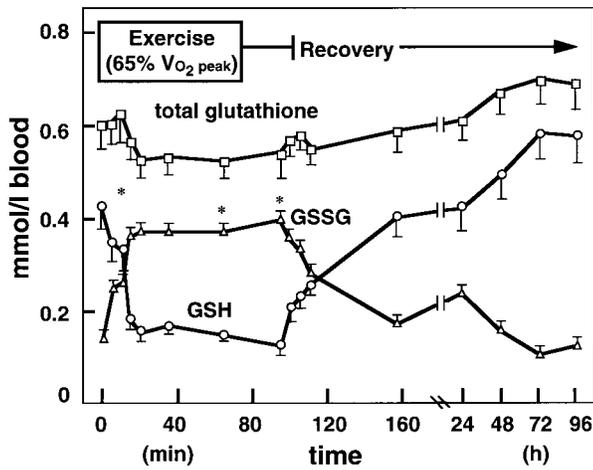
FIGURE 5. Mean (±SE) human blood glutathione oxidation induced by bicycle ergometry and postexercise recovery (67). GSH, reduced glutathione; GSSG, oxidized glutathione; $\dot{V}O_2$peak, peak oxygen uptake. *Significantly different from resting values, $P < 0.05$. Reproduced with permission from Gohil et al (67).

erythrocytes, cardiac muscle cells, and skeletal muscle cells, research has shown that excess intracellular GSSG is pumped out of the cell by an energy-dependent mechanism (79–81); such efflux of GSSG from oxidatively stressed tissues may account for the exercise-induced decrease in the total glutathione pools of these tissues, as discussed above.

In rats, the abilities of exhaustive physical exercise and intraperitoneal hydroperoxide injection to cause oxidative stress have been compared (82); an exhaustive treadmill run increased amounts of GSH in deep vastus lateralis muscle, but hydroperoxide injection had no effect. In that study, hepatic GSH amounts were not influenced by exercise. The exercise bout increased the amount of skeletal muscle GSSG, but no such effect was observed in the liver. Based on the muscle GSH oxidation results, it was concluded that the bout of exercise induced more oxidative stress than did the bolus of oxidant.

EXERCISE-INDUCED CHANGES IN PROTEIN SULFHYDRYLS

Most exercise studies related to thiols have investigated GSH metabolism. GSH is present in the cell in the mmol/L range, but this accounts for only half of all cell thiols. Using monobromobimane as the thiol probe we observed that only 50% of total thiols are accounted for by GSH (83). One component of proteins that are highly susceptible to oxidative damage are the sulfhydryl residues. Oxidative protein damage is widespread within the body at rest; Floyd (84) estimated that, even at rest, 0.9% of the total oxygen consumed by a cell contributes to protein oxidation. Most of this damage is irreparable. Thus, byproducts of such damage are either stored or degraded. Proteins that have been damaged by reactive oxygen are highly susceptible to proteolytic cleavage. Exercise-induced protein oxidation studies have used mainly the formation of carbonyls as the marker (8), and information regarding the effect of exercise on protein sulfhydryls is scanty.

Protein sulfhydryls are highly unstable and, if not adequately processed, are likely to be oxidized during sample processing or assays. Thus, it is essential that membrane-permeable thiol probes be used to process protein sulfhydryls in the intact tissue. Sulfhydryl status and oxidative damage of microsomes derived from skeletal muscle in swim-exercised rats were studied, but this work (85) suffers from the fact that microsomal sulfhydryls were reacted with a thiol-detecting agent, dithionitrobenzoic acid, only after tissue homogenization, differential centrifugation, and denaturation with detergent. It is unlikely that the redox state of microsomal sulfhydryls would resist all such tissue-processing procedures. Swim exercise induced oxidative lipid damage, but this was accompanied by an increase in tissue sulfhydryls (85). Seward et al (86) observed in rats that although exhaustive exercise is followed by a remarkable decrease in heart nonprotein GSH content, there are no changes in tissue total soluble thiol status. More recently, plasma protein-bound thiols were studied in healthy young men who participated in a full marathon race (42.195 km) after 6 mo of training (87). Plasma protein thiol concentrations were markedly decreased immediately after the race, suggesting that protein sulfhydryls were oxidized during the competition. In contrast with the oxidation of blood GSH during exercise, which is known to recover rapidly during the postexercise period, plasma protein thiol concentrations remained low even after 24 and 48 h of postmarathon recovery.

Acute exercise has been shown to markedly decrease phosphofructokinase activity, the rate-limiting enzyme of glycolysis, in white (rich in type IIb fiber) and red (rich in type IIa fiber) gastrocnemius muscles. In both old and young age groups, such an effect appears to depend on the muscle fiber type [IIb > IIa > I (soleus)]. The degree of phosphofructokinase down-regulation was inversely related to the activities of superoxide dismutase and glutathione peroxidase in the tissue, indicating that the down-regulation process may have been driven by secondary changes triggered as a response to oxidative stress. Mammalian phosphofructokinase is rich in exposed sulfhydryl groups that make the enzyme more prone to oxidant attack (88). It has also been observed that in cells derived from skeletal muscle, certain membrane K^+ transport proteins are highly sensitive to oxidant exposure (89).

MANIPULATION OF TISSUE GLUTATHIONE

Factors such as the central role of GSH in the antioxidant network and the enhanced oxidation of tissue GSH during exercise have generated considerable interest in searching for effective pro-GSH nutritional supplements. Numerous studies have sought to evaluate the efficacy of various thiol agents to bolster tissue GSH, and attempts have been made to determine whether dietary selenium may enhance GSH-dependent antioxidant defenses by increasing tissue glutathione peroxidase activity.

Selenium

GSH-dependent enzymatic metabolism of hydroperoxides involves glutathione peroxidase activity. Selenium acts as a cofactor of glutathione peroxidase and thus selenium is necessary to maintain full-strength GSH-dependent antioxidant defense (90). Dietary deficiency of selenium remarkably lowers tissue glutathione peroxidase activity and thus makes the tissue more susceptible to oxidative damage. In one study, chronic dietary deficiency of selenium did not affect the body weight or endurance capacity of rats even though the activity of selenium-dependent glutathione peroxidase in the liver and skeletal muscles decreased by >80% (91). The authors explained that

weakening of the glutathione peroxidase–dependent antioxidant protection may have activated alternative antioxidant pathways as a compensatory response. However, selenium-independent glutathione peroxidase activity was not increased in selenium-deficient rats. The liver vitamin E content of selenium-deficient rats was significantly decreased, indicating enhanced consumption of this lipophilic antioxidant. Selenium supplements appeared to have a sparing effect on the dietary requirement for vitamin E.

In another study (61), dietary selenium deficiency remarkably decreased selenium-dependent glutathione peroxidase activity in the liver and skeletal muscle. Selenium-independent glutathione peroxidase activity was barely affected by the dietary restriction. Thus, dietary selenium insufficiency may limit GSH-dependent tissue antioxidant defense. The effects of dietary selenium supplementation (0.5 ppm) and of selenium deprivation on the liver, muscle, and blood of rats in a swimming protocol have also been tested (92). Tissue glutathione peroxidase activity sensitively responded to dietary selenium status, and selenium deficiency was associated with increased oxidative lipid damage in tissues. In a double-blind human study, 180 μg selenomethionine supplement every day for 10 wk increased plasma glutathione peroxidase activity but had no effect on physical performance (49).

Riboflavin

Regeneration of GSH from GSSG inside the cell depends on GSSG reductase activity in the presence of NADPH, and activity of this enzyme requires the tissue to have adequate riboflavin status. Thus, nutritional vitamin B supply may influence tissue GSH redox cycle activity. Because GSSG reductase activity is sensitive to the riboflavin status of the tissue, tissue GSSG reductase activity is often used as a marker of riboflavin availability in tissues (93). Regular physical exercise decreases riboflavin excretion and enhances its retention in tissues (93, 94); in this way more riboflavin retained in tissues can favorably influence GSSG reductase activity.

Strategies to increase cellular glutathione

Enhancing tissue GSH reserve in mammals is a challenging task, particularly because GSH per se is not available to most tissues when administered orally or injected intraperitoneally. The net amount of tissue GSH depends primarily on 2 factors: GSH neosynthesis and GSH regeneration from GSSG (95). Synthesis of GSH in the cell is rate-limited by the availability of the amino acid cysteine, which, in its reduced form, is highly unstable. Thus, >90% of cysteine in the human circulation is present in the oxidized cystine form (96). In cell culture media, this amino acid is present only in the cystine form. Thus, one strategy to enhance intracellular GSH is to improve cysteine availability within the cell (Figure 3). For example, addition of a reducing agent such as β-mercaptoethanol to cell culture media will enhance cell GSH content (95, 97). Other ways to boost GSH synthesis in the cell include enhancing the activity of GSH-synthesizing enzymes by gene transfer. In *Escherichia coli* these specific genes have been isolated and used to transform the wild strain to overexpress the synthetase enzymes (98). Similar work with mammalian cell systems has been performed (99, 100), but at present this approach is far from being clinically relevant.

Glutathione esters

The cell GSH reserve may be directly elevated by administering GSH esters. Both GSH mono(glycyl)esters and GSH diethyl esters have been used for this purpose (101). Unlike GSH itself, which is a lipophobic molecule that cannot penetrate the cell membrane, esterified GSH is lipophilic and thus membrane permeable. However, using such esters to boost the tissue GSH pool is not without limitations. For example, metal ion contamination of GSH monoesters remarkably decreases the capacity of the compound to serve as a GSH-delivering agent. In addition, certain forms of esterified GSH, such as the GSH dimethyl ester, appear to be toxic to mice (102). Furthermore, some esters are deesterified by esterase activity in the plasma and lose their ability to permeate the cell membrane. Levy et al (102) reported that GSH diethyl ester may be the GSH delivery agent of choice, especially for those species (eg, humans, not rats or mice) that lack GSH diester α-esterase in the plasma (102). GSH diester has been found to be nontoxic to mice and hamsters, but has yet to be tested in vivo in humans. Therefore, although the use of such forms of esterified GSH is appealing, at present it may be premature to run clinical trials to test the efficiency of such esters in managing exercise-induced oxidative stress.

N-acetyl-L-cysteine

Improving cysteine availability for the biosynthesis of GSH is the most extensively studied approach for enhancing the cell GSH pool. Among the agents tested are *N*-acetyl-L-cysteine (NAC), lipoic acid, cysteamine, and 2-oxothiazolidine 4-carboxylate. Other reducing agents, such as dithiocarbamate, β-mercaptoethanol, and dithiothreitol, may also improve GSH synthesis through the extracellular reduction of cystine to cysteine. NAC and α-lipoic acid have generated the most interest because of their proven clinical safety features and efficacy in vivo (16, 95, 96, 103–110). Oral NAC (2-mercapto-propionyl glycine) was found to elevate GSH in the plasma and bronchoalveolar lavage fluid (107). NAC effectively controls perturbations in the thiol redox status after acetaminophen toxicity and has been successfully used for clinical purposes (105).

α-Lipoic acid

α-Lipoic acid is also known as thioctic acid, 1,2-dithiolane-3-pentanoic acid, 1,2-dithiolane-3-valeric acid, and 6,8-thioctic acid. At physiologic pH, lipoic acid is anionic and in this form it is commonly called lipoate. As early as the 1950s, α-lipoate was seen as an essential cofactor in oxidative metabolism (16, 83, 95, 109–111). Biologically, lipoate exists as lipoamide in ≥5 proteins, where it is covalently linked to a lysyl residue. Four of these proteins are found in α-ketoacid dehydrogenase complexes: the pyruvate dehydrogenase complex, the branched-chain keto-acid dehydrogenase complex, and the α-ketoglutarate dehydrogenase complex. Three of the lipoamide-containing proteins are present in the E2 enzyme dihydrolipoamide S-acetyltransferase, which is different in each of the complexes and is specific for the substrate of the complex. One lipoyl residue is found in protein X, which is the same in each complex. The fifth lipoamide residue is present in the glycine cleavage system (111).

Lipoic acid is detected in the form of lipoyllysine in various natural sources (112). When expressed as weight per dry weight of lyophilized vegetables, the relative abundance of naturally existing lipoate was found to be spinach > > broccoli floral buds > tomato fruit > garden peas and Brussels sprouts > rice bran. Lipoyllysine concentrations were not detectable in acetone powders of banana, orange peel, soybean, and horseradish. In animal tissues, the abun-

dance of lipoyllysine in bovine acetone powders was determined to be kidney > heart > liver > spleen > brain > pancreas > lung. Concentrations of lipoyllysine in spinach and bovine kidney were 3.15 ± 1.11 and 2.64 ± 1.23 µg/g dry wt, respectively (112).

Lipoate has generated considerable clinical interest as a thiol-replenishing and redox-modulating agent (16, 17, 83, 95). Recently, it was also observed that lipoate treatment selectively facilitates the death of cancer cells by potentiating the inducible activity of caspase 3, also known as death protease (113). A unique property of lipoate is that it is a "metabolic antioxidant"; enzymes in human cells accept it as a substrate for reduction. Accordingly, supplemented lipoate is promptly taken up by cells and reduced to dihydrolipoate at the expense of cellular-reducing equivalents such as NADH and NADPH (114, 115). As more of these reducing equivalents are used, the rate of cellular metabolism is expedited to cater to the enhanced demand (115). Dihydrolipoate is a powerful reducing agent (Table 1) with many antioxidant properties that mediates the pro-GSH effects of lipoate (Figure 3). Thus, a unique property of supplemental lipoate is harnessing the power of the cell's own metabolic processes for its recycling and potency. In addition to its remarkable effect in strengthening antioxidant defenses, lipoate is known to promote the efficiency of glucose uptake by cultured skeletal muscle cells at a magnitude comparable to that of insulin (116, 117). Interestingly, lipoate was shown to retain its ability to stimulate glucose uptake even where L6 myotubes were insulin resistant (117). Whether such an insulin-mimetic property of lipoate influences muscle bioenergetics during exercise is an open question.

After being enzymatically generated inside the cell, dihydrolipoate rapidly escapes to the extracellular culture medium (114). To improve retention in cells, we recently modified the α-lipoic acid molecule to confer a positive charge at physiologic pH (118); the protonated form of the new molecule is called LA-Plus. We found the uptake of LA-Plus by human T cells to be higher than the uptake of lipoate. In addition, amounts of DHLA-Plus, the corresponding reduced form of LA-Plus, were several times higher in cells treated with LA-Plus than were amounts of dihydrolipoate in cells treated with lipoate. Furthermore, on a concentration basis, LA-Plus was more biologically potent than lipoate (118, 119). These promising results set the stage for further testing of the possible beneficial role of LA-Plus as an antioxidant supplement.

THIOL MANIPULATION AND EXERCISE

Glutathione, *N*-acetylcysteine, and lipoic acid supplementation in animal exercise studies

Although supplemented GSH is poorly available to tissues, 2 brief studies claimed that exogenous GSH remarkably increases endurance to physical exercise in mice (120, 121). These results are surprising, but because no biochemical measurements were reported one should not conclude that exogenous GSH enhanced endurance by minimizing oxidative stress. Moreover, the exercise protocol used in these studies may have been too brief ($\approx$5 min) to induce oxidative stress. More complete studies are necessary to explain how exogenous GSH may enhance exercise performance.

To test the time-dependent distribution of intraperitoneally administered GSH, we administered the thiol (1 g/kg body wt) to male Wistar rats (76). Injection of GSH solution resulted in a rapid appearance ($>10^2$ times 0.5 h after administration) of glu-

tathione in the plasma. After such a response, a rapid clearance of plasma total glutathione was observed. Twenty-four hours after the injection, plasma total glutathione was restored to the preinjection control concentration. Excess postinjection plasma GSH was rapidly oxidized, as detected by the presence of GSSG. Supplemented GSH was not available to tissues such as the liver, skeletal muscles, lung, kidney, or heart. However, after repeated injection of GSH for 3 consecutive days, total amounts of glutathione in blood and kidney increased. No such effect was observed in the liver, red gastrocnemius muscle, mixed vastus lateralis muscle, heart, or lung (76).

Rats treated with a single injection of GSH or NAC (1 g/kg body wt) were subjected to exhaustive treadmill exercise 0.5 h after the intraperitoneal injection (76). The exercise induced GSH oxidation in several tissues, including the skeletal muscle, lung, blood, and plasma. GSH supplementation did not protect against exercise-associated changes in GSH status and lipid peroxidation in the liver, skeletal muscle, heart, and lung of rats. Exercise caused blood GSH oxidation and NAC supplementation protected against such oxidation. NAC also appeared to protect against exercise-induced perturbation of GSH redox status in the lung. Neither GSH nor NAC supplementation had any influence on endurance during an exhaustive long distance treadmill run (76). In a separate study in which GSH supplementation was studied in endurance-trained rats, such supplementation appeared effective in decreasing exercise-induced leakage of mitochondrial superoxide dismutase protein from tissues to the plasma (122).

The influence of supplementation with GSH (1 g/kg body wt), NAC (1 g/kg), or vitamin C (0.5 g/kg) for 1 wk on exercise-induced blood GSH oxidation was also investigated in rats. GSH supplementation had no significant effect, but both NAC and vitamin C supplementations partially decreased blood GSH oxidation after treadmill exercise (72).

Although it has been argued that lipoate supplementation may improve mitochondrial function by facilitating the activity of lipoyllysine-containing enzymes (123), there is no evidence to support this contention. The first study testing the possible effects of oral α-lipoic acid supplementation as well as the effect of a single bout of strenuous exercise and endurance exercise training on the lipoyllysine content of skeletal muscle and liver tissues in rats was reported recently (124). Incorporation of the lipoyl moiety to tissue protein was not increased by dietary lipoate. Interestingly, endurance exercise training markedly increased lipoyllysine content in the liver at rest, and a bout of exhaustive exercise also increased hepatic lipoyllysine content. A significant interaction between exhaustive exercise and training in increasing tissue lipoyllysine content was evident. In vastus lateralis skeletal muscle, training did not influence tissue lipoyllysine content. A single bout of exhaustive exercise, however, clearly increased the amount of lipoyllysine in the muscle. Comparison of tissue lipoyllysine data with results for free or loosely bound lipoate showed a clear lack of association between the 2 apparently related parameters. Thus, the tightly protein-bound lipoyllysine pool in tissues is independent of the loosely bound or free lipoate status in the tissue (124).

In the first study of the efficacy of α-lipoate supplementation in exercise-induced oxidative stress, Khanna et al (125) examined the effect of intragastric lipoate supplementation (150 mg/kg body wt for 8 wk) on lipid peroxidation and glutathione-dependent antioxidant defenses in the liver, heart, kidney, and skeletal muscle of male Wistar rats. Lipoate supplementation

significantly increased total glutathione in liver and blood, a finding consistent with previous in vitro experiments (95) that shows that lipoate supplementation may indeed increase amounts of glutathione in certain tissues in vivo. Lipoate supplementation, however, did not affect the total glutathione content of organs such as the kidney, heart, and skeletal muscles. A lipoate-supplementation-dependent increase in the hepatic glutathione pool was associated with increased resistance to lipid peroxidation. This beneficial effect against oxidative lipid damage was also observed in the heart and red gastrocnemius skeletal muscle. Lower lipid peroxide amounts in certain tissues of lipoate-fed rats suggest a strengthening of the antioxidant defense network in these tissues (125).

Glutathione deficiency models

Tissue GSH synthesis depends on dietary amino acid supply. Thus, food deprivation decreases tissue GSH content and refeeding corrects such an effect. Leeuwenburg and Ji (126) showed that exhaustive treadmill exercise tends to further lower the amount of GSH in the liver of food-deprived rats. This effect was more prominent in rats that were refed and had higher baseline amounts of GSH in the liver. When the supply of dietary amino acids, particularly cyst(e)ine, is inadequate, tissues may be expected to suffer from higher susceptibility to oxidative stress as a result of lowered GSH content as well as decreased activity of antioxidant enzymes (127).

The hypothesis that endogenous tissue GSH status is critically important in protecting against exercise-induced oxidative stress and thus relates to endurance for exhaustive exercise was tested in GSH-deficient rats (76) prepared by intraperitoneal injection of L-buthionine-[S,R]-sulfoximine (101). This treatment selectively inhibits the first enzyme of GSH synthesis, γ-glutamylcysteine synthetase (glutamate–cysteine ligase), and turns off intracellular GSH synthesis. The treatment resulted in 1) an ≈50% decrease in the total glutathione pools of the liver, lung, blood, and plasma and 2) an 80–90% decrease in the total glutathione pools of the skeletal muscle and heart. Compared with that in the placebo-treated controls, endurance to exhaustion of GSH-deficient exercising rats was reduced by half. Results of this experiment indicate a crucial role of endogenous GSH in circumventing exercise-induced oxidative stress and in determining exercise performance (76). In another study, depletion of GSH by diethyl maleate significantly decreased the swim performance of rats (128).

The effect of a more long-term GSH deficiency was studied in mice subjected to a swimming protocol (129). Global GSH deficiency was induced by the intraperitoneal injection of L-buthionine-[S,R]-sulfoximine combined with supplementation of 20 mmol L-buthionine-[S,R]-sulfoximine/L in drinking water for 12 d. GSH contents in the plasma, liver, kidney, heart, and skeletal muscles were decreased by 65%, 77%, 85%, 90%, and 93% of the control values, respectively. A longer exercise bout lasting 4–6 h was tested and no effect of GSH deficiency on swim endurance was observed in these mice. A marked decrease in the amount of hepatic GSH was observed during exercise. Consistent with the observation of Sen et al (76), exercise-induced oxidative lipid damage was greater in GSH-deficient mice, particularly in skeletal muscle. Increased tissue lipid peroxidation in a GSH-deficient state agrees with the hypothesis that GSH plays a central role in the antioxidant network (Figure 1) and that impaired GSH defense weakens the efficacy of lipid-phase antioxidants. Leeuwenburgh and Ji (129) showed that GSH defi-

ciency also influences the activity of antioxidant enzymes. For example, GSH deficiency was associated with decreased glutathione peroxidase activity in the liver, but activities of GSSG reductase and glutathione transferase were elevated. GSH deficiency may also influence oxidative metabolism in tissues. It has been consistently observed that the activity of the mitochondrial enzyme citrate synthase in the liver, kidney, and skeletal muscle is lower in GSH-deficient tissues (129, 130).

Human supplementation studies

The effect of oral NAC supplementation on rapid blood GSH oxidation in subjects who performed identical maximal bicycle ergometer exercises 3 wk apart was tested in our laboratory (70). Before the second test, the men took NAC tablets (4×200-mg tablets/d) for 2 d and an additional 800 mg on the test morning. In all experiments, blood samples were drawn before, immediately after, and 24 h after tests. NAC supplementation increased the free radical scavenging capacity of human plasma. Rapid blood GSH oxidation associated with the maximal test was markedly attenuated by NAC supplementation, indicating that the treatment spared exercise-associated perturbation of blood thiol redox status. In a separate study, trained athletes were orally supplemented with a combination of 1 g GSH and 2 g vitamin C daily for 7 d to test the possible effect of this treatment on exercise-induced blood GSH oxidation (72). In all 5 men studied, linearly progressive-intensity treadmill exercise induced blood GSH oxidation. The magnitude of this effect ranged from a 34% to a 320% increase in blood GSSG over preexercise concentrations. The antioxidant supplementation protocol was effective in completely protecting against blood GSH oxidation induced by exercise.

Regulation of neutrophil function

Strenuous physical exercise is known to trigger an acute-phase immune response, a stereotyped series of host defense reactions involving reactive oxygen species comparable to that induced by bacterial infection (131, 132). As are inflammation and infection, exercise is associated with increased body temperature, an increase in serum cytokines (eg, interleukin 1 and interferon α), an increased number of circulating leukocytes, and priming of neutrophils for an oxidative burst response (131). A neutrophil oxidative burst serves as a physiologic defense mechanism for killing pathogens and healing wounds, but during exhaustive exercise it may also contribute to muscle damage (133, 134). Although a short bout of physical exercise may stimulate the mobilization of leukocytes and result in their increased concentration in the circulation, long-lasting high-intensity exercise is known to cause leukopenia. Under such conditions, leukocytes marginate into sites of tissue damage (135, 136). Oxidative bursts at these sites may induce further damage; such effects may be mediated by potent oxidants such as hypochlorite or hydrogen peroxide that are known to be produced during the burst activity.

Atalay et al (137) showed that changes in rat neutrophil function induced by exhaustive exercise may be regulated by whole-body GSH status. These researchers tested the effects of intraperitoneal GSH supplementation and carried out experiments using GSH-deficient exercised rats. Leukocyte margination induced by exhaustive exercise was prevented by GSH supplementation. In addition, global tissue GSH deficiency induced by L-buthionine-[S,R]-sulfoximine treatment suppressed neutrophil oxidative bursts induced by opsonized zymosan (137). This suggests that endogenous GSH regulates exercise-induced immune responses. The effect of NAC
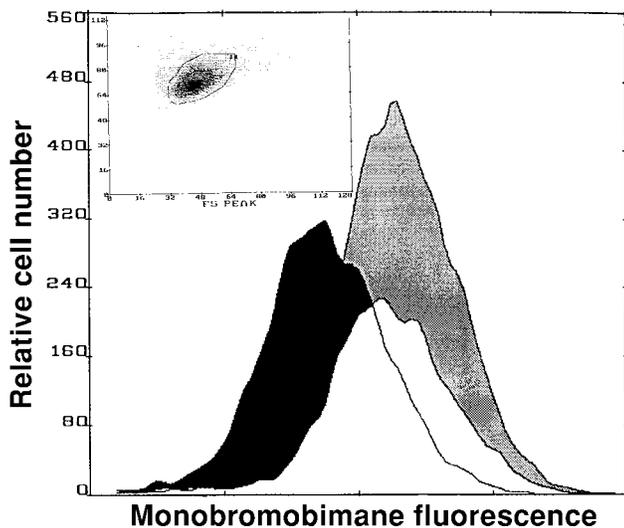
FIGURE 6. Lipoate pretreatment of cells bypasses glutamate-induced cell thiol depletion. Data are presented as cell population fluorescence distribution plots of flow cytometry data. The *x* axis is a log scale representing cell thiol fluorescence signal as detected by treating intact cells with 40 μmol monobromobimane/L. A decrease in this signal, ie, a shift toward the origin, represents decreased cell thiol content. The ordinate represents relative cell count. Human Jurkat T cells were either grown in regular culture medium (shaded), treated for 18 h with an excess of 5 mmol glutamate/L (filled), or treated for 18 h with an excess of 5 mmol glutamate/L in the presence of 100 μmol lipoate/L (open). Monobromobimane-loaded cells were excited by using a 20-mW powered ultraviolet line of a Innova 90-4 argon ion laser (Coherent, Palo Alto, CA) set at 350 nm in a EPICS Elite flow cytometer (Coulter, Miami). Fluorescent emission from cellular sulfhydryl-reacted bimane was recorded by using a 450-nm band pass filter. A morphometrically homogenous viable cell population, typically representing ≈90% of the total population, was gated (inset). Data were collected from ≥10000 cells at a flow rate of 250–300 cells/s. For more details, *see* reference 83.

supplementation on exercise-induced priming of human neutrophils has also been studied (138). NAC supplementation did not increase blood GSH but improved the capacity of blood plasma to scavenge peroxyl radicals. Exercise was followed by neutrophil priming as detected by an oxidative burst response to treatment with autologous serum-coated zymosan (139). Although NAC did not influence blood GSH concentrations, it markedly suppressed zymosan-induced oxidative bursts in postexercise neutrophils. Thus, the ability of thiol supplements to regulate exercise-induced neutrophil priming in humans was evident.

Muscle fatigue

In addition to the experiments on exercise-induced blood GSH oxidation and neutrophil response discussed above, research has been conducted on NAC and muscle fatigue (139, 140). In experimental studies with excised rat diaphragm muscle, NAC treatment inhibited acute fatigue (139); in human studies by Khawli and Reid (139) and Reid et al (140), the effect of intravenous infusion of NAC on the development of acute muscle fatigue after electrical stimulation was investigated. The surface of the motor point of the tibialis anterior muscle was stimulated at 40–55 mA in pulses lasting 0.2 s. Fatigue was produced by using repetitive tetanic stimulations at 10 Hz or 40 Hz frequencies. During 10 Hz contractions, NAC significantly increased force production and

NAC pretreatment improved human limb muscle performance during fatiguing exercise. In this well-controlled human study in which the endpoints and antioxidant treatment strategy were well designed, the efficacy of NAC was convincingly shown.

HUMAN DISEASE

Reactive oxygen species are known to contribute to a wide variety of human pathologies, and thus the question may be raised as to whether exercise-induced oxidative stress contributes to the development of these diseases. This issue should be of particular concern for persons already suffering from or at high risk of developing health disorders that may be caused in part by oxidative stress. For example, oxidative modification of LDL is a major factor in the pathogenesis of atherosclerosis. Because physical exercise can cause plasma lipid peroxidation and protein oxidation, should individuals who are currently developing or are at high risk of developing atherosclerosis be more cautious about exercise-induced oxidative stress? Questions of this type, which warrant high priority, have been poorly addressed so far.

In one of the first studies of its type, Laaksonen et al (74) studied exercise-induced oxidative stress in young men with type 1 diabetes. Oxidative stress has been implicated in microvascular complications of diabetes and markers of such stress are elevated in diabetic patients even without complications. Exercise led to marked blood GSH oxidation and plasma lipid peroxidation in these men, but physical fitness seemed to protect against exercise-induced oxidative stress.

Disturbances in tissue thiol homeostasis have been linked to several human health disorders (95). For example, persons infected with HIV usually have decreased concentrations of acid-soluble thiols, particularly cysteine and GSH, in their plasma and leukocytes (96, 110, 141–143). Decreased thiol status is a typical marker of oxidative stress and thus HIV infection and oxidative stress appear to be related. The same research group that made such findings also noted a significantly higher plasma concentration of glutamate in HIV-infected and cancer patients (144–146). Availability of cysteine is the rate-limiting step in cellular GSH synthesis. Because of its marked instability in the reduced form, >90% of extracellular cysteine is present as cystine (96). Glutamate competitively inhibits cystine uptake by cells (**Figure 6**); in this way, substrate for GSH synthesis within the cell is limited in the presence of high concentrations of extracellular glutamate. In Jurkat T cells we observed that supplementation of the culture medium with 5 mmol glutamate/L results in a 50% reduction in cellular GSH (83). Similar decreases in cell thiol content in the presence of 10 mmol/L excess extracellular glutamate were observed in C6 glioma cells, where excess glutamate markedly decreased cellular GSH content, led to accumulation of oxidants inside the cell, and proved to be cytotoxic after 18–24 h of exposure (147).

Studies using flow cytometry showed that lipoate treatment can increase cellular GSH in a dose-dependent manner from 10 to 100 μmol/L (83). Interestingly, lipoate can completely bypass glutamate inhibition of GSH synthesis (Figure 6). From results in our laboratory we developed the following mechanistic explanation: *1*) when cells are treated with lipoate, lipoate rapidly enters the cells and is reduced to dihydrolipoate; *2*) dihydrolipoate expelled from the cell to the culture medium reduces extracellular cystine to cysteine; and *3*) cysteine can bypass glutamate inhibition of cystine uptake and provide sufficient substrate for cellular GSH synthesis (95). Because these effects of lipoate are

observed at concentrations ≤ 100 μmol/L, they should be considered clinically relevant. In the C6 glial cell model, treatment with 100 μmol lipoate/L abolished glutamate-induced cytotoxicity and markedly prevented the loss of cellular thiols (147).

SIGNAL TRANSDUCTION

To develop an understanding of the molecular bases of oxidant and antioxidant action with respect to the development of human disease, current research has focused on redox-sensitive signal transduction. Redox gene therapy has been recognized to have remarkable clinical potential (148), and at least 2 redox-sensitive transcription factors, nuclear factor κB (NF-κB) and activator protein 1 (AP-1), are well defined (15–17). These transcription factors may be involved in the pathogenesis of several human diseases, including AIDS and cancer. For example, the long terminal repeat region of HIV-1 proviral DNA contains 2 NF-κB binding sites (149, 150); DNA binding of NF-κB can activate HIV transcription. Thus, strategies to suppress NF-κB–dependent transactivation may be important for delaying the onset and progression of AIDS (110). The intracellular thiol redox state was observed to be a major regulator of NF-κB response and thiol agents such as lipoate, NAC and anethole dithiolethione are effective in inhibiting activity of this transcription factor induced by various cytokines, phorbol ester, or oxidants (16, 151–153). In trying to understand the mechanism underlying oxidant-induced NF-κB activation, we observed that oxidant-induced changes in intracellular calcium status play a critical role (152). Both lipoate and NAC diminished oxidant-induced elevation in intracellular calcium ion concentration (16, 152). In a previous section of this work we reported that skeletal muscle GSH status is tightly regulated by the state of physical activity; for example, exhaustive exercise causes tissue GSH oxidation, exercise training may increase muscle total glutathione, and activity restriction may decrease muscle total glutathione (8). We also observed that such changes in the amount of total glutathione or the redox state of glutathione may markedly regulate inducible NF-κB activity in L6 myoblasts derived from skeletal muscle (154).

Among the clinically relevant thiol-replenishing agents tested so far, NAC and lipoate undoubtedly hold the most promise. Although in many respects the effect of NAC is similar to that of lipoate, much higher concentrations of NAC are required to produce comparable effects. Normally, under experimental conditions, 10–30 mmol NAC/L is used for effects related to the regulation of cell signaling, but just 1 mmol lipoate/L is clearly effective in suppressing NF-κB activation in response to diverse stimuli (16, 152). In Jurkat T cells, although 100 μmol NAC/L failed to enhance cellular GSH, lipoate could do so in a dose-dependent manner from 10–100 μmol/L as observed by flow cytometry (83). Much of these concentration differences may be explained by the mode of lipoate action. As discussed previously, lipoate harnesses the metabolic power of the cell to continuously regenerate its potent reduced form. In this way, the reduced lipoate pool can be continuously renewed at the expense of the cell's metabolic power. For NAC, this is not possible, and thus higher concentrations are necessary. Furthermore, a study that directly compared the efficacy of NAC and lipoate with respect to NF-κB–mediated gene expression found that 200 μmol lipoate/L resulted in a 40% decrease in HIV-1 p24 antigen expression in tumor necrosis factor α–stimulated OM 10.1 cells latently transfected with HIV-1, but that 10 mmol NAC/L was required to produce comparable effects (155).

CONCLUSIONS

Biological thiols play a central role in regulating cell function (15–17, 95, 156). In oxidative stress, thiols serve not only as a cornerstone of the antioxidant defense network but also as regulators of molecular responses to oxidants. Tissue thiol homeostasis is markedly influenced by the state of physical activity, as shown by evidence related to the various specific effects of physical training and restriction of activity on different tissues. In addition, blood GSH oxidation appears to be a consistent response to physical exercise in humans. Proper functioning of GSH-dependent antioxidant defenses requires adequate constituent amino acid, selenium, and riboflavin supply in the diet. Although GSH administered per se may not be available to tissues, factors that improve cysteine delivery to tissues may increase GSH. Among the various compounds tried so far for their efficacy to enhance tissue GSH, NAC and lipoate hold the most promise, particularly in humans. Lipoate enjoys a major advantage over NAC primarily because exogenous lipoate, but not NAC, is accepted as a substrate by human oxidoreductase enzymes. As a result, lipoate can continuously use the cell's metabolic power for its own recycling to the potent dithiol form. In the presence of NADH or NADPH, lipoate can be enzymatically reduced in human tissues to dihydrolipoate, which is a potent antioxidant, a strong reducing agent, a modulator of redox-sensitive signal transduction, and a markedly effective agent in enhancing cellular thiol status. The recently designed molecule LA-Plus (118, 119) appears to be a more potent analogue of α-lipoic acid and thus should be tested further for efficacy in animal and human studies.

DIRECTIONS FOR FUTURE RESEARCH

- Advanced techniques such as flow cytometry should be used to study the thiol status response in human blood cells. This will provide information from various cell subsets, not just a mean value as is usually obtained from biochemical measurements.
- Reactive oxygen species influence cell signaling processes that are implicated in a wide range of human health disorders. The study of such redox-sensitive molecular targets in response to physical exercise and elucidation of the efficacy of different antioxidant nutrients in favorably influencing such molecular mechanisms should receive high priority.
- Human studies investigating the effect of physical exercise on indexes of oxidative stress in women, children, and the elderly are lacking.
- Development of nutritional strategies to elevate tissue GSH represents an important and challenging task.
- α-Lipoic acid is the only thiol antioxidant supplement that is recycled from its oxidized form to the corresponding reduced form by enzymes of the human cell. Most of the current information regarding this unique antioxidant nutrient has been obtained from cell culture studies. More animal and human studies are necessary to optimize daily nutritional requirements.

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