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EXTRACELLULAR THIOLS AND THIOL/DISULFIDE REDOX IN METABOLISM

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■ **Abstract** Many proteins present on cell surfaces and located in extracellular fluids contain cysteine and methionine residues that are subject to oxidation. These proteins, which include transporters, receptors, and enzymes, respond to variations in the extracellular thiol/disulfide redox environment. Changes in activity of these proteins can alter the ability of organs to function normally and influence processes such as nutritional absorption, secretory function, neurotransmission, and susceptibility to toxicants. In addition, extracellular redox can regulate tissue homeostasis through effects on cell proliferation, differentiation, apoptosis, and immune function. Consequently, extracellular redox can have important influences on health status and disease states and thus could be a target for nutritional interventions.

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INTRODUCTION

The function and stability of macromolecules such as proteins, nucleic acids, and lipids is influenced by the environment in which they reside, and the extracellular compartment differs significantly from the intracellular compartment. Proteins present in extracellular fluids and on the surfaces of cells are susceptible to oxidation, especially of sulfur atoms in cysteine (Cys) and methionine (Met) residues. Control of the redox states of the sulfur atoms is critical for normal function, which includes important processes of digestion and nutrient absorption. Moreover, dietary components can affect systems that regulate extracellular redox. This review summarizes recent advances in the understanding of extracellular thiol homeostasis and regulation of thiol/disulfide redox state in extracellular fluids.

Biologic Importance of Sulfur

Sulfur exists stably in multiple oxidation states, which makes it a versatile component in biological systems. The most highly active and most reduced form of sulfur in biomolecules is the thiol (-SH), present in the amino acid cysteine (Cys). Cys is present in the active site of many proteins and in protein motifs that function in enzyme regulation, protein trafficking, control of gene expression, and receptor signaling. The body's supply of Cys is from protein in the diet and through the transulfuration of the essential dietary amino acid, Met. Accumulating data suggest that reversible oxidations of sulfur residues are common and fundamentally important in control of cell functions (19, 34, 90, 97). Most of the literature addresses oxidation of thiols in proteins, which can be described as "sulfhydryl switching" mechanisms. However, interconversions of Met and methionine sulfoxide also serve this function. The sulfur in Met is present as a thioether ($-CH_2-S-CH_3$), which is inherently less reactive but nonetheless critical for many protein functions and subject to oxidation (Figure 1).

The vast literature on biological thiols is focused on thiols in cells, but the intracellular volume of the body is only two thirds of the total; the remaining one third consists of extracellular fluid such as plasma, interstitial fluids, lining fluids of the lung and oral cavity, biliary and pancreatic secretions, and intestinal lumen contents. The extracellular fluids are involved in delivering and exchanging nutrients, maintaining tissue homeostasis, signaling between cells, and protecting against oxidative stress. In vitro data indicate that variations in extracellular redox state have significant impact on critical cell functions such as proliferation, differentiation, and apoptosis. Normally, the in vivo thiol/disulfide balance is tightly controlled, but there is variation in response to disease, environmental factors, and

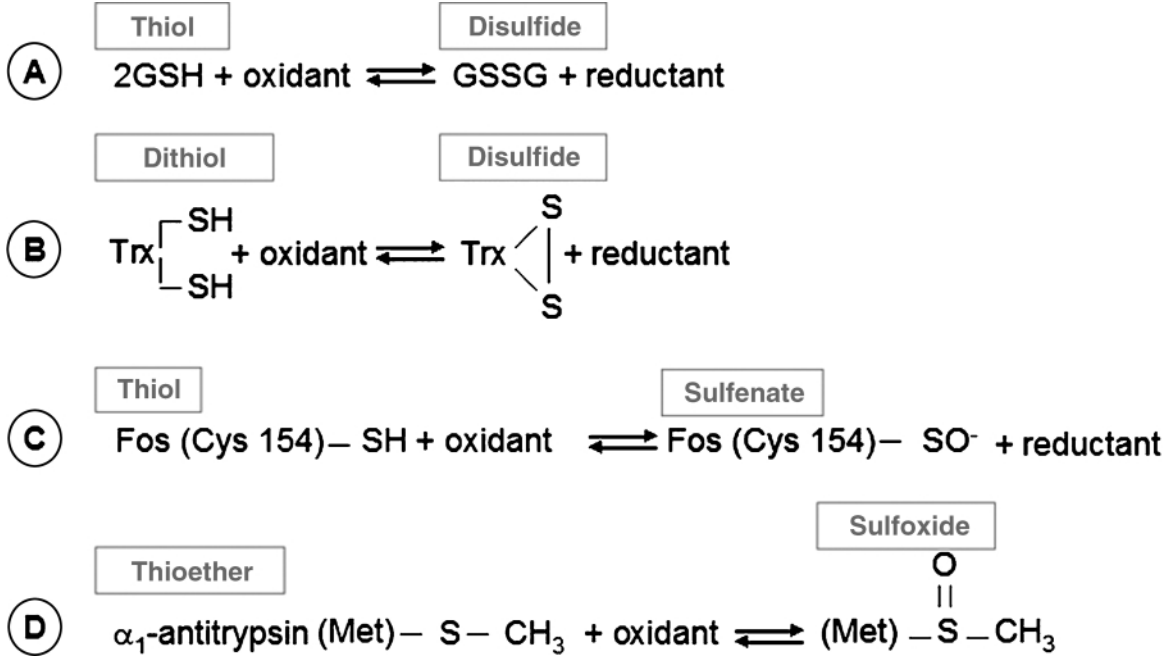

Figure 1 Reversible oxidation-reduction of sulfur atoms in cysteine and methionine provides a common mechanism for control of physical and functional properties of proteins. Many amino acids can undergo oxidation, and sulfur atoms can be oxidized to a number of different oxidation states, but only Cys and Met undergo reversible oxidations and these involve only a limited number of oxidation states. Both low-molecular-weight thiols and protein thiols undergo reversible oxidation to form disulfides, as illustrated by glutathione (GSH) oxidation to form glutathione disulfide (GSSG) (*A*). In this reaction, two compounds are linked together to form one chemical. Proteins containing two thiols can be oxidized to form an internal disulfide, as illustrated by thioredoxin (*B*). Oxidation of cysteine 154 of the DNA-binding site of c-Fos results in a cysteine sulfenate, which does not bind DNA. This latter structure does not readily occur in aqueous solution and in this case is stabilized by the protein structure (*C*). The thioether of methionine can be reversibly oxidized to a sulfoxide, as occurs in α_1-antitrypsin (*D*).

nutrition. Because nutrition can impact the regulation of thiols and thiol/disulfide redox in the extracellular compartments, and oxidation of extracellular redox has been linked to chronic disease and toxicity, the accumulating data indicate that dietary control of extracellular redox may be a useful parameter for interventions to improve health and assess disease.

Oxidative Stress and Disease

Although oxidants are constantly generated for essential biologic functions, excess generation or an imbalance between oxidants and antioxidants can produce a common pathophysiologic condition termed oxidative stress. Oxidative stress contributes significantly to age-related diseases such as atherosclerosis, chronic lung disease, age-related macular degeneration, and Alzheimer's disease (40, 85, 108, 134). Numerous reviews are available that address nutrition, reactive oxygen species, and oxidative stress (36, 64); consequently, in the present paper we focus on thiols, disulfides, and redox balance. Redox state will be defined using the Nernst equation, where the reducing force of the thiol/disulfide couples is quantitatively described in terms of the tendency to accept or donate electrons (59, 120).

CELLULAR REDOX

A complex interaction exists between extracellular redox control and cellular redox control, with extracellular redox ultimately dependent upon the cellular redox state. Two principal systems maintain cellular thiol/disulfide redox state: glutathione (GSH) and thioredoxin (Trx). These systems are complementary but also have overlapping activities that provide a partial redundancy in their functions. GSH is a low-molecular-weight thiol present at millimolar concentrations in cells whereas Trx is a small protein present at micromolar concentrations. GSH is well suited for functions in detoxifications, interorgan cysteine homeostasis, and redox control. Trx contains a dithiol motif at its active site, which is ideally suited for reduction of protein disulfides, sulfoxides, and sulfenic acids, but also is capable of peroxide elimination and is used for deoxyribonucleotide biosynthesis.

Redox Variation Within Cells

Cellular GSH/glutathione disulfide (GSSG) redox state varies in cells in association with proliferation, differentiation, and apoptosis (Figure 2). Proliferating cells have GSH/GSSG values ranging from −260 mV to −230 mV (59, 66). The mechanistic link between redox and cell cycle has not been completely elucidated, but a redox effect has been identified. For instance, in normal fibroblasts, mRNA levels of *gro*, a gene associated with proliferation, were correlated with the redox environment and the proliferative state of the cells (41). The GSH pool becomes oxidized (−220 mV to −190 mV) during growth arrest, either due to

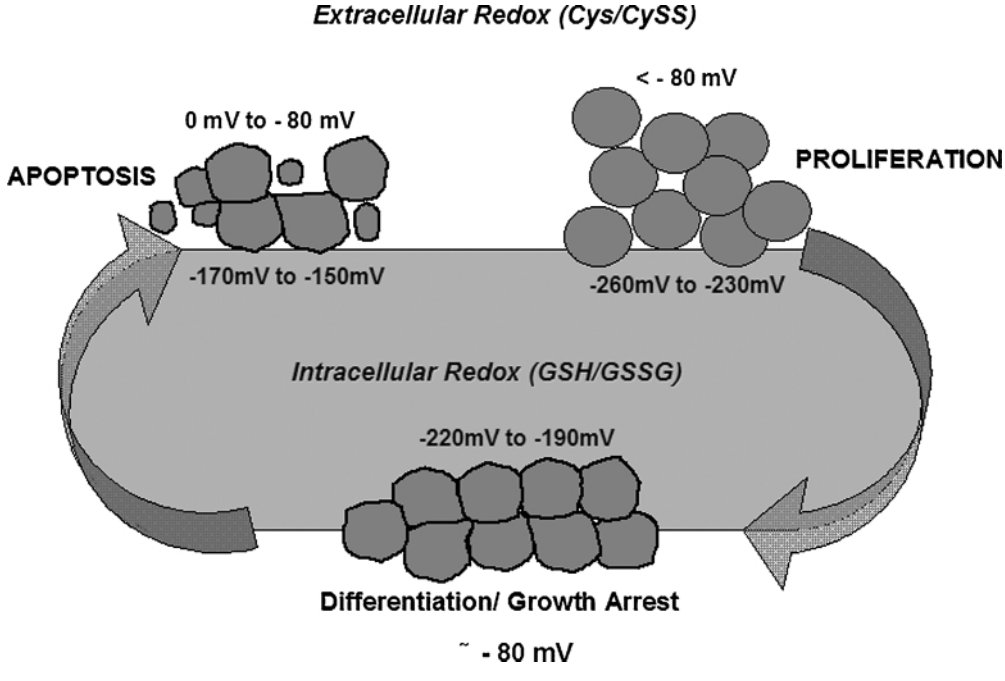

Figure 2 Cellular and extracellular redox are oxidized in association with the progression of cells from proliferation to differentiation, growth arrest, and apoptosis. In mammalian cells in vitro, the intracellular GSH/GSSG redox is most reduced during proliferation. This redox state becomes more oxidized in differentiation and subsequent apoptosis. The extent of oxidation is sufficient to regulate protein function by sulfur-switching mechanisms. The extracellular Cys/CySS redox is considerably more oxidized than cellular GSH/GSSG, but its redox state follows this pattern, being most reduced during proliferation and most oxidized during apoptosis. In vivo studies of fasting and refeeding effects on redox state in intestinal epithelia show that the cellular redox state is most oxidized in the fasting state and becomes reduced upon refeeding. These changes correlate with changes in growth indices, as expected from the in vitro data. Cys/CySS, cysteine/cystine; GSH, glutathione; GSSG, glutathione disulfide.

differentiation or contact inhibition (42, 66). In normal fibroblasts, an increase in confluency led to an oxidation of the redox state (34 mV) and cessation of proliferation, whereas fibrosarcoma cells maintained a reduced state and continued to proliferate despite an increased culture density (42). In human bronchial epithelial cells, growth arrest followed a decrease in intracellular GSH and Cys levels (4). An oxidation of intracellular GSH/GSSG redox as well as lowered intracellular GSH and GSSG concentrations were observed in contact-inhibited, nondividing human retinal pigment epithelial (hRPE) cells (54). These nondividing hRPE cells were more susceptible to tert-butyl hydroperoxide (tBH)-induced apoptosis. During apoptosis, GSH export from cells is activated and the redox state is further oxidized to between −170 mV and −150 mV (11, 53).

Of considerable interest relative to nutrition, a Cys deficiency limits cell growth and is sufficient to result in a marked oxidation of GSH/GSSG redox (89). This oxidation indicates that dietary availability of sulfur amino acids may directly determine thiol/disulfide balance in cells. Because Cys is required for protein synthesis, the associated redox change may provide a central mechanism for coupling dietary sulfur amino acid availability to cell growth and tissue homeostasis.

Compartmentation of Redox

The cellular redox measurements largely reflect the cytoplasmic compartment. The restricted movement of biomolecules imposed by both the plasma membrane and intracellular membranes creates multiple compartments with different redox states. Based upon available data, which are limited for some organelles, the redox of the secretory pathway and lysosomes appears to be relatively oxidizing while that of the nucleus is relatively reducing (44, 141). The mechanisms for controlling redox in different compartments has been studied very little, though it appears likely that regulation occurs through a combination of oxidoreductases and transport systems (Figure 3).

Redox-Regulated Cell Surface Proteins

Cell surface proteins such as receptors, transport proteins, and enzymes that contain thiol moieties can be influenced by redox regulation (Figure 4). Cell-surface glycosylphosphatidylinositol-anchoring proteins and lipid raft moieties have been suggested to be direct targets of oxidative stress. Oxidation of proteins in these rafts induces clustering through disulfide bond cross-linking of cell-surface proteins and consequent activation of Src family protein tyrosine kinases (97). Iesaki & Wolin (45) found that extracellular thiol oxidation activates a redox-regulated coronary vasodilator mechanism that involves the inhibition of Ca^{2+} influx. Treatment of isolated endothelium-removed bovine coronary arteries with the thiol oxidant diamide caused relaxation, presumably due to the obstruction of Ca^{2+} influx (45).

Protein tyrosine phosphatases are intracellular redox-sensitive proteins that can be reversibly oxidized and act as regulators of cell surface receptor tyrosine kinases

(34). N-acetylcysteine (NAC), Cys, and GSH have the ability to modulate the redox state of extracellular cysteine residues of target proteins such as mitogen-activated protein kinase (MAPK), but may also exert effects intracellularly by acting upon the redox-sensitive phosphatases. In Caco-2 cells, MAPK p44 phosphorylation was dependent on modulation of the extracellular cysteine/cystine (Cys/CySS) redox where, in the most reduced conditions, MAPK p44 was phosphorylated (Y. Nkabyo, T. Ziegler & D.P. Jones, unpublished observations). NAC, Cys, and GSH addition to bovine and human chondrocyte serum promoted survival through phosphorylation of extracellular signal-regulated kinases (ERK) (72). Additional research is needed to identify critical redox-sensitive thiols in these systems, and to determine whether the redox gradient between extracellular and intracellular spaces is a component of the dynamic function of these signaling mechanisms.

EXTRACELLULAR REDOX

Recent in vivo data have shown that the redox state of thiol/disulfide pools in human plasma varies little between individuals and that cultured cells and perfused tissues regulate the redox state in extracellular compartments. Thus, mechanisms to control extracellular redox exist, and variation in the function of these systems can be expected to contribute to the susceptibility of tissues to oxidative stress during aging and disease.

Major differences between cellular and extracellular compartments exist both in terms of the concentrations of thiol/disulfide systems and their relative redox states. Perhaps the most frequently recognized difference is that the major low-molecular-weight thiol/disulfide system in cells, GSH/GSSG, is principally in the reduced form, while the major low-molecular-weight system in the extracellular compartment, Cys/CySS, is principally in the disulfide form, cystine.

Plasma

Although only a minor component relative to protein and cysteine, GSH is the most extensively studied extracellular thiol/disulfide component in the plasma. The plasma functions in nutrient supply and in maintenance of redox state of proteins on the extracellular surface of cells (Figure 4). In the human plasma, GSH is generally in the range of 2–4 μM, with a redox value in healthy adults of -137 ± 9 mV, which is considerably more oxidized than in cells and tissues (60). Decreases in the plasma GSH pool have been found to be associated with human immunodeficiency virus (HIV) infection (8), prematurely born infants (52), protein energy malnutrition (114), and alcoholic cirrhosis (14). Decreased plasma GSH implies a decrease in systemic GSH availability, which may increase the susceptibility of compromised individuals to oxidative stress.

Cys availability is often a limiting factor for the rate of GSH synthesis (16, 87). Indeed, during injury, oxidative stress, and metabolism of foreign compounds by GSH conjugation, increased Cys is required to maintain GSH synthesis (39,

81). Most cell types cannot synthesize Cys from Met and have little uptake of CySS; therefore, uptake of Cys is critically important to support cellular protein synthesis and GSH needs (3, 48, 119). Cys concentration is decreased in fasting and starvation, and individuals with hepatic cirrhosis have hypocysteinemia due to impaired conversion of Met to Cys (14). Oral supply of Cys is able to augment Cys and results in a rapid increase in plasma Cys concentration (60). Supplementation with NAC, a cysteine precursor, was shown to protect against apoptosis (82, 118). In murine T-cell hybridoma cells, this protection was independent of intracellular GSH, occurring also with the nonphysiologic D-isomer, which cannot be converted to GSH (62). Taken together, these observations show that Cys has multiple protective functions, probably including support of protein synthesis, maintenance of GSH concentrations, and maintenance of cellular redox state.

The major extracellular low-molecular-weight thiol pool is Cys/CySS. Given that GSH cannot be hydrolyzed to yield Cys within cells, a mechanism is needed to convert cellular GSH to Cys to maintain Cys supply in the postabsorptive state. Thus, a major function of extracellular GSH is not as an antioxidant, but as a substrate for γ-glutamyl transferase, the first enzyme in a pathway to provide Cys from GSH degradation. This can be illustrated schematically in terms of the different sources of plasma Cys following a meal where, initially, plasma Cys is maintained by dietary protein intake (Figure 5). In the postabsorptive state, plasma Cys rapidly decreases and hepatic GSH release and hydrolysis in the kidney

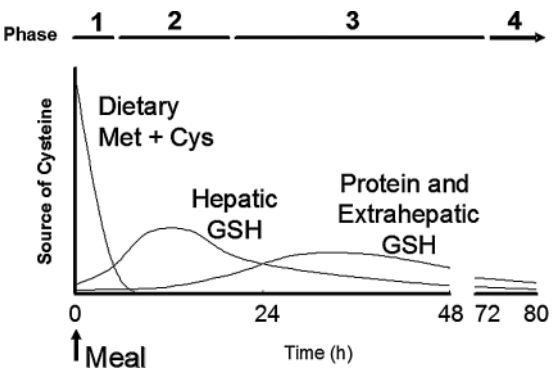

Figure 5 The source of plasma cysteine varies as a function of time after eating a meal containing sulfur amino acids. During the first hours following a meal, the plasma supply of cysteine (Cys) is largely from dietary Cys and methionine (Met). During this phase, stimulated glutathione (GSH) synthesis results in an accumulation of hepatic GSH. GSH is continuously released by the liver, and under postabsorptive conditions, this release provides a main source of plasma Cys because plasma GSH is rapidly degraded in the kidney. At subsequent times when hepatic GSH levels fall, other tissues, especially skeletal muscle, maintain plasma Cys through GSH release.

take over as the major source of circulating Cys. The hepatic content of GSH is approximately equal to the recommended dietary allowance for sulfur amino acids, so that by the end of the first day without dietary intake, supply of GSH from extrahepatic sources must increase in importance as a source for circulating Cys.

The Cys/CySS redox pool is present at concentrations much higher than that of the GSH/GSSG redox pool. The predominant form of Cys in the blood plasma is the oxidized disulfide form, CySS. Cys is in the range of 8–10 μM in the plasma, while CySS is found at concentrations >40 μM (60). The redox value for the Cys/CySS pool is -80 ± 9 mV in healthy adults, a level that is significantly more oxidized than the GSH pool (60).

Trx and Trx reductase (TrxR) are secreted by different cell types and may play a role in the extracellular environment (96, 129). Oxidative stress was shown to enhance secretion of Trx (67). In addition, oxidative stress–related diseases such as rheumatoid arthritis (56), asthma (142), hepatitis C (131), HIV (94), and steatohepatitis (130) showed increased plasma Trx, signifying Trx as an important component of the extracellular thiol environment.

Intestine

Intestinal utilization of extracellular GSH is vital for prevention of oxidative injury to the intestine and elimination of harmful toxicants from the diet. The intestinal epithelium has three sources of GSH: endogenous synthesis, biliary supply, and dietary intake. The lumen receives millimolar concentrations of hepatic GSH from biliary secretion (21), and fresh fruits and vegetables as well as many types of meat provide a rich source of dietary GSH (61). Transport of GSH across the basolateral membrane of enterocytes provides another possible source for luminal GSH. Interestingly, the redox state of the luminal Cys/CySS pool is more reduced than the redox state of the GSH/GSSG pool. Although incompletely defined, the data suggest that the mechanism of luminal GSSG reduction involves Cys efflux and uptake of CySS (18). This mechanism, which has been termed a cysteine-cystine shuttle, provides a means to maintain thiol/disulfide redox state at about -150 mV in the lumen.

GSH is also required for normal intestinal growth and function. For example, a study using buthionine sulfoximine, which inhibits GSH synthesis, demonstrated that GSH is required for normal intestinal function (84). In inflammatory bowel disease, there is decreased activity of enzymes involved in GSH synthesis accompanied by a decreased availability of Cys for GSH synthesis (125). Another study showed a redox imbalance caused by a decline in GSH inhibits proliferation of colon carcinoma cell line Caco-2 (99). Suppression of mucosal cell turnover induced by peroxidized lipids in rat small intestine was reversed with GSH supplementation (136).

The luminal Cys/CySS pool plays a key role in growth and absorption of redox-sensitive nutrients (18). During absorption, Cys functions as a reductant of iron and selenium, thereby regulating absorption of these nutrients (121, 122, 137).

Both Cys and GSH may also function in the maintenance of mucus fluidity (128), which may aid movement of ingesta through the lumen and minimize physical trauma to the epithelial layer.

Multiple studies have demonstrated the importance of Cys and GSH in intestinal cell cycle control. A study in a human colon carcinoma cell line, HT29 cells, showed that extracellular Cys deficiency caused an 89 mV oxidation of the GSH/GSSG redox state (89). The extent of this oxidation in HT29 cells is sufficient to result in a >100-fold change in the ratio of redox-sensitive dithiol/disulfide moieties in proteins. Supplementation with Cys (136) attenuated the suppression of cell proliferation and restored intracellular GSH redox status (99). However, other studies indicate that extracellular Cys and CySS may be able to modulate proliferation independent of intracellular GSH. Cys and CySS addition to Caco-2 cells induced cell proliferation without an increase in intracellular GSH (100). Such an effect could be mediated by direct effects on receptors within the plasma membrane or by increased cellular Cys.

Lung

The extracellular thiol environment is an important determinant of tissue function in the lung. The lung epithelium must provide an effective barrier against oxidants, other reactive chemicals, and microorganisms, while at the same time allowing facile diffusion of blood gases. GSH is an important component of these defenses, with the concentration of GSH in the extracellular lining fluid (ELF) reaching values of greater than 400 μM (110), a value comparable to those in some cell types.

Extracellular GSH has been found to protect against hyperoxia-induced injury in isolated rat type II cells (115). This protection suggests that preservation of GSH in the ELF is important, and efforts to therapeutically restore GSH redox state in the ELF have been made using aerosolized preparations of GSH and the GSH precursor NAC. These preparations have focused on cystic fibrosis (CF), where a change in extracellular redox has been well documented (38). The CF transmembrane conductance regulator (CFTR) channel, which is mutated in CF, is permeant to GSH, and this characteristic could explain the observation that there is an extracellular deficiency of GSH and an oxidized redox state of GSH/GSSG (38, 116). However, the limited sampling capability in human disease and the complexity of the animal models have limited understanding of the mechanistic details.

Recent studies have shown that the incidence and severity of acute respiratory distress syndrome is greatly amplified in chronic alcoholics, and this observation has been linked to a decrease in alveolar GSH (28). Chronic alcoholics have significantly decreased ELF concentration of GSH and increased GSSG compared with nonalcoholics (92). These studies have been corroborated in rats, where chronic ethanol ingestion decreased the alveolar ELF GSH (33). Chronic alcohol consumption is known to be associated with decreased antioxidant levels, and cirrhosis from

alcohol abuse has been associated with hypocysteinemia and decreased circulating GSH concentrations (135). Consequently, the heightened susceptibility of the lungs of alcoholics to oxidant-induced injury may be a reflection of a systemic deficiency in GSH homeostasis.

Central Nervous System

Studies of extracellular redox in the central nervous system have been largely limited to the cerebrospinal fluid in the context of disease processes thought to involve oxidative stress. Oxidative stress is implicated in many neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), Parkinson's disease, and Alzheimer's disease. Oxidative stress is associated with a compromised GSH system in brain in such neurological diseases and probably occurs both due to enhanced reactive oxygen species (ROS) generation and impaired GSH homeostasis. Cellular GSH is present in the central nervous system in millimolar concentrations and is essential for the cellular detoxification of ROS (6, 101, 113). An unusual feature of GSH homeostasis in the brain is that increased extracellular GSH appears more likely to be deleterious rather than protective because a more reduced state can potentiate excitotoxicity.

GSH is released from neurons via a calcium-dependent, depolarization-enhanced process, but the role of this extracellular GSH remains unknown (144). At low micromolar extracellular concentrations, GSH (which contains glutamate linked to cysteine through the γ-carboxyl) may bind to its own receptors or displace glutamate from ionotropic receptors, thus modulating depolarization and excitatory neurotransmission. Conversely, at higher concentrations, GSH may increase *N*-methyl-D-aspartate (NMDA) receptor responses by interacting with its redox sites (112).

Regan et al. (111) demonstrated that extracellular GSH in the micromolar range enhanced neuronal loss due to energy depletion by making the extracellular redox state more reduced, which results in an increased activation of the NMDA receptor. Additionally, neuronal death after 20–25 hours exposure to 6–9 μM NMDA was not altered by 10–100 μM GSH, but was greatly augmented by 300–1000 μM GSH (111, 112). Cysteine also produces excitotoxic effects and perhaps is involved in the reducing environment of neurons in these diseases (105).

Toghi et al. (132) reported that, in the cerebrospinal fluid (CSF) of untreated patients with Parkinson's disease, the concentration of GSSG was significantly decreased, and the GSH/GSSG ratio was increased, suggesting that extracellular GSH/GSSG redox state is more reduced in untreated Parkinson's disease patients. Furthermore, in sporadic ALS patients the CSF GSH/GSSG ratio was approximately threefold higher than that of controls (133). Additional studies of GSH and thiol/disulfide redox in extracellular fluids of the central nervous system are clearly warranted. The accumulating data that implicate oxidative stress provide a logical basis for antioxidant supplementation. However, to the extent that excitotoxicity contributes to the pathology and is enhanced by a reduced state, nutritional changes

that produce a more reduced state may not be desirable. Available scientific data are inadequate to reach a conclusion concerning the suitability of interventions designed to enhance thiol redox state in the brain, and additional mechanistic and nutritional interventional studies are needed.

Other Systems

Skeletal muscle participates in regulation of the extracellular GSH content and therefore could be important in regulation of the Cys pool and the extracellular redox status. Plasma GSH is mostly derived from hepatic efflux, but in hepatectomized rats GSH was maintained in the plasma at 50% of the control value. In rats administered phorone, a compound that depletes GSH conjugation, GSH in the peripheral plasma was not higher than concentrations in the plasma from the portal vein, which indicated that the depleted liver was not releasing GSH (9). Thus, in addition to the normal supply of GSH to the plasma from liver, extrahepatic tissue also functions in the export and maintenance of plasma GSH. The available evidence suggests that skeletal muscle is a major source of GSH when hepatic stores are decreased (9, 70).

Limited data are available on interstitial fluid GSH content in muscle. In situ microdialysis in skeletal muscle showed that extracellular GSH levels increased 100% at the end of the ischemic period compared to preischemic values. Immediately after reperfusion, GSSG levels increased 100%, which indicated that transient hypoxia in muscle tissue decreases intracellular GSH stores by leakage to the extracellular environment, resulting in an increased risk for oxidative injury upon reperfusion (126). Thus, the results with interstitial fluid in muscle support the conclusions from other tissues showing that redox of extracellular fluid is rapidly responsive to changes in tissue oxidative stress and therefore may provide a useful material to monitor nutritional effects on tissue thiol and redox balance.

GSH is also present in all other body fluids for which measurements have been made, including saliva, sweat, milk, seminal fluid, and urine. This implies that tissue release is a ubiquitous process. Moreover, extracellular GSH is ubiquitously detected in media of cultured cells. This common occurrence of extracellular GSH is likely due to release by the widely distributed multidrug-resistant transporters (78, 109).

IN VITRO STUDIES OF EXTRACELLULAR THIOL/DISULFIDE REDOX

The extracellular thiol environment influences cell proliferation, differentiation, and apoptosis. In a study to determine optimum conditions for growth in a biofermenter, Hwang & Sinskey studied major parameters controlling cell density for more than 20 mammalian cell lines (43). With controlled oxygen partial pressure and pH, cell density was maximal with a redox potential of about −60 mV, and

redox potential was predominantly determined by thiol content of the medium (43). Although this study did not distinguish between effects on cell proliferation and cell death rates as determinants of cell density, recent studies show that cell proliferation rate is dependent upon the redox state of Cys/CySS in the culture medium (57, 58, 89), and that a more reduced redox state is associated with resistance to apoptosis (55).

In a study of proliferation in human Caco2 cells, Jonas et al. (58) varied extracellular thiol/disulfide redox state over a physiologic range (−150 mV to 0 mV) of reducing to oxidizing conditions by altering the extracellular Cys relative to the CySS concentrations in cell medium. In the most oxidizing conditions (0 mV), proliferation was the lowest; it was 100% higher at the most reduced condition (−150 mV). This study showed that extracellular redox influenced growth without detectable effect on cellular GSH levels, suggesting that extracellular redox may affect growth factor signaling. Studies with insulin-like growth factor 1 (IGF-1) and epidermal growth factor (EGF) showed that these growth factors stimulated proliferation in the oxidizing conditions (0 to 80 mV), but had no effect in the most reduced conditions (−150 mV). Moreover, IGF-1 and EGF, as well as glutamine (Gln) or keratinocyte growth factor, induced a shift in extracellular redox state to a value similar to that found in plasma in vivo (57, 58). Thus, the data suggest a redox feedback loop controlling cell proliferation in which proliferation is optimized by redox state and growth factors stimulate cells to control extracellular redox.

The interaction of cellular and extracellular redox was further studied in Caco-2 cells allowed to grow to confluency without added growth factors. As cell density became maximal with contact inhibition and differentiation markers increased, cellular redox became oxidized. A 28-mV oxidation of extracellular Cys/CySS was associated with this cellular oxidation (98). The underlying mechanisms of this interaction between extracellular redox control and cell proliferation rate remain unknown, and additional studies are needed to determine whether these changes are exerted through cell surface receptors and/or involve regulation of gene expression.

Effects of extracellular redox on gene expression were studied in aortic endothelial cells in experiments to determine whether the observed variation in plasma redox with aging could contribute to key early events of atherosclerosis (Y. Go, K. Openo & D.P. Jones, manuscript in preparation). A comparison of the oxidized redox state common to older individuals with that of young, healthy adults showed that oxidation of extracellular thiol/disulfide redox state of Cys/CySS enhanced cellular ROS generation, and activated the transcription factor nuclear factor-κB (NF-κB). Expression of adhesion molecules controlled by NF-κB, including ICAM-1, PECAM-1, and P-selectin, was increased and adhesion of monocytes was stimulated (Figure 4). Thus, the results show that the extracellular redox state of Cys/CySS can modulate inflammatory events of early atherosclerosis.

Lymphocytes require a reducing environment for optimal activation and proliferation. Lymphocytes lack an efficient system of CySS uptake, whereas they readily take up Cys. Extracellular Cys content is closely linked to intracellular GSH

content (31, 47, 48). Following stimulation with lipopolysaccharide, mouse spleen lymphocytes increased their capacity to transport Cys, but not CySS, through the ASC system (Na^+-dependent neutral amino acid transport system). In addition, in these activated lymphocytes, Cys increased cellular GSH content (48). Interestingly, macrophages have the ability to uptake CySS and secrete Cys, regulating the microenvironment and subsequent function of lymphocytes in the vicinity (31, 119, 124, 139). Antigen-presenting dendritic cells also have the ability to provide a reducing extracellular environment needed for T lymphocyte activation by release of Cys and Trx (3). Extracellular Trx can have a synergistic role on the mitogen- or cytokine-induced proliferation of lymphocytes (50, 138).

Additional studies indicate that extracellular Trx can be important in regulating cell functions. In normal liver cells, secretion of Trx was accompanied by growth inhibition and morphological changes, whereas in hepatocarcinoma cell line HepG2, there was no secretion of Trx or growth inhibition (138). A B-cell lymphoma line responded to exogenous thiols and Trx with an increase in proliferation, which indicates that the function of extracellular Trx may be complex and vary for different cell types. TrxR was secreted in vitro from both normal and transformed cells and was inducible by physiologic stimuli such as IFN-γ, lipopolysaccharide, and interleukin 1α. Exogenous Trx protected lymphoid cells against TNF-α or hydrogen peroxide–mediated cytotoxicity and prevented apoptosis caused by depletion of Cys and GSH (49).

Numerous studies have documented that extracellular GSH and Cys-producing precursors of GSH protect against apoptosis. Most of these studies have been interpreted in terms of the need to maintain cellular GSH for antioxidant defenses. While this interpretation is correct, some studies suggest additional mechanisms are involved. For instance, the D-isomer of N-acetylcysteine, which is not a precursor for GSH synthesis, provided the same protection against activation-induced apoptosis as found for the L-isomer (62). This finding suggests that the thiol/disulfide redox state may influence activation of apoptosis. Similarly, in human retinal pigment epithelial cells, exogenously added GSH inhibited oxidant-induced upregulation of the Fas death receptor system even though GSH is not transported into these cells. These studies taken together suggest that in addition to mechanisms that support cellular antioxidant defenses, variation in extracellular redox can have critical functions in regulation in cell growth and survival.

PERTURBATIONS IN EXTRACELLULAR REDOX

Aging

Several studies are available to show that extracellular thiol/disulfide redox state becomes oxidized with aging. Among the changes that are well documented are decreased GSH, increased CySS, and increased total homocysteine, which is predominantly present in disulfide forms (17, 63). Glutathione concentration in the cerebral spinal fluid of humans also decreases with age (17, 63). However, it is

difficult to determine whether these changes represent a decline of antioxidant defenses or excessive production of ROS leads to aging and age-associated disease states. In older subjects compared to younger subjects, whole blood GSSG was increased and in the plasma GSH was lower, and the GSH/GSSG redox was more oxidized (117). There was also a decrease in Cys and an increase in CySS concentration. Oxidation of the Cys redox pool correlated with a decrease in body cell mass and plasma albumin, which suggests that such an oxidation may contribute to the loss of body cell mass (35, 37). However, therapeutically, albumin levels in humans increased with NAC treatment, indicating loss of albumin can be rectified (7, 35).

In a study that examined redox of individual thiol/disulfide couples in human plasma, a linear oxidation of about 0.2 mV/year was found for Cys/CySS, whereas the GSH/GSSG redox was maintained until 45 years of age, after which it was found to rapidly decline by 0.7 mV/year (63). This implies that the two pools are regulated separately in vivo. Moreover, because the cell growth characteristics in vitro are responsive to Cys/CySS and GSH is reflective of detoxification function, the results suggest that the two redox pools may provide distinct information on in vivo function. Specifically, the Cys/CySS pool may provide an indicator of growth and repair functions, whereas the GSH/GSSG pool may reflect detoxification functions. If so, the measurement of these distinct redox pools could provide useful biomarkers for human aging and disease risk.

Lifestyle Consequences

Accumulating evidence indicates that lifestyle choices such as smoking, alcohol intake, and exercise alter extracellular thiol homeostasis. Cigarette smoke contains compounds (e.g., acrolein) that react with GSH, as well as ROS, which oxidize GSH to GSSG (110). Interestingly, lung GSH levels are elevated in chronic smokers, suggestive of altered gene expression of glutamate cysteine ligase (also γ-glutamylcysteine synthetase), the rate-limiting enzyme in GSH synthesis (107). In a study of redox in subjects >50 years of age, the Cys/CySS plasma redox in smokers (–64 $\pm$ 16 mV) was more oxidized than in nonsmokers (–76 $\pm$ 11 mV; $p < 0.001$), with decreased Cys in smokers (9 $\pm$ 5 μM) compared to nonsmokers (13 $\pm$ 6 μM; $p < 0.001$). The GSH/GSSG redox was also more oxidized in smokers (–128 $\pm$ 18 mV) than in nonsmokers (–137 $\pm$ 17 mV; $p = 0.01$), and GSH was lower in smokers (1.8 $\pm$ 1.3 μM) than in nonsmokers (2.4 $\pm$ 1.0; $p < 0.005$). Although the oxidation of GSH/GSSG can be explained by the role of GSH in detoxification of reactive species in smoke, the more extensive oxidation of the Cys pool shows that smoking has additional effects on sulfur amino acid metabolism. Smoking causes an oxidation in both the plasma GSH/GSSG redox and the Cys/CySS redox and seems to significantly decrease the plasma cysteine pool size (91).

As indicated above, the deleterious effects of alcohol are generally considered to be due to oxidative stress, with an increase in free radical production accompanied

by a decrease in antioxidant systems. There is a decrease of hepatic GSH with chronic alcohol abuse, and in cirrhotic patients, there is a decrease in plasma, erythrocyte, and liver Cys, CySS, and GSH (2, 14, 75, 76). In alcoholics who abstain from alcohol, treatment with GSH improves the GSH concentration in the plasma (77). In addition, alcoholic cirrhotics have a decrease in total GSH concentration in the alveolar ELF accompanied by an oxidation of this pool (increase in GSSG) (28, 92). The decrease in the GSH concentration in ELF could provide a mechanistic link to susceptibility of acute respiratory distress syndrome in patients with a history of chronic alcohol abuse (92).

Both acute and chronic exercises provide an oxidative stress that modifies extracellular redox. Trained subjects maintain higher plasma levels of GSH than untrained subjects. This effect appears to be due to an increased GSH export from trained muscle into the plasma. In subjects 36–53 years of age, long-distance runners were observed to have a significant increase in GSH levels compared to sedentary controls, but in a younger group, 18–35 years of age, there was no difference (69). In trained soccer players, blood malondialdehyde levels were significantly lower and their plasma thiols were significantly elevated compared to controls, indicating that conditioned players were under less oxidative stress than controls. Studies carried out on beagle dogs trained over one year demonstrated increased GSH concentrations in the trained muscle and in the plasma, but no change in GSSG compared to untrained dogs. This indicates that the GSH system of the skeletal muscle adapts to training and is able to produce more GSH and therefore export more GSH to the plasma (83).

A converse effect can be seen with acute exercise. With sporadic, acute exercise in rats, liver GSH was depleted to 20% of sedentary controls (71, 106). There was a transient increase in plasma GSH levels followed by a linear decline of GSH, which continued for several hours following cessation of exercise (71, 106). Similarly, healthy, untrained human females had significantly increased levels of protein-bound sulfhydryl groups after running for 30 minutes, and a transient decrease occurred in the erythrocyte low-molecular-weight thiols. While the latter levels returned to baseline after 2 hours, the former remained unchanged after 24 hours. Additional studies show that even in trained individuals, such as marathon runners, oxidized plasma proteins temporarily increase after acute exercise (46). Thus, physical exercise causes short-term oxidation of thiol/disulfide pools in both untrained and conditioned individuals, but long-term exercise conditioning results in improved resistance to oxidation of these pools under normal conditions.

Disease

Considerable evidence shows that oxidative stress is associated with disease, but uncertainty remains concerning whether this is a cause or consequence of specific disease processes. Numerous reviews that summarize this literature are available, e.g., in cardiovascular disease (12), neurodegenerative disease (23), cancer (68), diabetes (74), age-related macular degeneration (10), and HIV infection (123). Enhanced generation of ROS and impaired antioxidant detoxification functions

contribute to an imbalance between oxidative and reductive reactions, which is reflected in the extracellular thiol/disulfide balance. Given the numerous processes that are dependent upon extracellular redox, variation in extracellular redox can be expected to contribute to disease. However, available evidence for specific contribution of extracellular thiol/disulfide redox is circumstantial; experimental design does not allow distinction between enhanced generation of ROS and altered thiol/disulfide redox as contributing factors. Indeed, these may be obligately coupled because thiols are used for detoxification of ROS and ROS are signaling agents for thiol homeostasis.

Consideration of the difference between 1-electron free radical processes and nonradical 2-electron transfers may be useful in delineating sequences of causality. Free radical processes cause a variety of oxidative changes in macromolecules that provide a biochemical footprint-reflecting mechanism. The difficulty in obtaining reliable clinical biomarkers for oxidative stress may be due to a varying contribution of free radical processes against the background of variation in diet-related changes in thiol/disulfide redox, which can also occur due to variation in sulfur amino acid intake and changes in expression of glutathione synthetic enzymes.

One of the most extensively studied conditions with regard to extracellular thiols is HIV infection. In symptom-free HIV-positive subjects, there is a systemic depletion of GSH (8, 22) due to a systemic decrease in synthesis (127). In addition, there is a decrease in Cys and CySS, which has been speculated to play a role in the pathogenesis of the disease (20, 22, 96). In contrast to these changes in low-molecular-weight thiols, plasma Trx levels were elevated with HIV progression (94, 95). In a small study, NAC supplementation was found to decrease Trx, increase Cys and GSH in the plasma, and increase survival. However, the widespread use of more potent antivirals has precluded unequivocal documentation that such supplementation has a general benefit in protection against HIV.

Several possible redox mechanisms may be involved in the pathogenesis of HIV infection. In vitro studies showed that thiol addition (GSH and NAC) to the media prevented virus replication (73). In addition, in vivo studies in mice showed that elevated plasma Trx efficiently blocks lipopolysaccharide-induced chemotaxis, an innate immune mechanism involved in compensating when adaptive immunity is compromised (95). Certain chemokines seem to specifically inhibit the ability of HIV to infect cells via a mechanism that blocks the interaction of the virus with the receptor. Cavallini & Alexandre reported that oral administration of NAC to healthy volunteers increased the ability of their peripheral blood mononuclear cells to release these protective chemokines (13).

The approaches used for the study of HIV highlight important paradigms for the study of the potential role of extracellular thiol/disulfide redox in disease. The effects of specific nutritional interventions on extracellular redox can be directly assessed along with disease progression in double-blind studies. At the same time, free radical processes can be measured in terms of relevant biomarkers, such as lipid peroxidation and protein oxidation products. The relevant contributions of 1-electron and 2-electron oxidative processes may differ at an individual level and may provide a useful basis for discriminating redox interventions targeted to use

of free radical scavengers to protect against the former and thiol precursors for protection against the latter.

Such distinction may be especially important in type 2 diabetes, where 2-electron oxidations may predominate in importance due to disruption of normal carbohydrate metabolism. Type 2 diabetes involves impaired glucose utilization with impaired NADPH supply and increased concentrations of reactive aldehydes. Extracellular perturbations of thiol/disulfide redox are common and studies have shown that plasma GSSG is increased, erythrocyte GSH is decreased, total GSH/GSSG pool size is decreased, and plasma GSH pool is oxidized (14, 15, 29, 93, 117). These changes are often accompanied by serious consequences such as diabetic retinopathy and severe edema in the extremities, indicating severe homeostatic imbalance. If the oxidation of thiol/disulfide redox occurs as a consequence of impaired glucose utilization and causes subsequent disease symptoms, nutritional interventions to improve thiol/disulfide redox may be a useful component of disease management.

DIETARY MANAGEMENT OF EXTRACELLULAR REDOX

Most of the diseases for which extracellular thiols or extracellular thiol disulfide redox state could be important are chronic and complex diseases, usually dependent upon multiple factors. As a consequence, definition of who might benefit from improved thiol or extracellular redox status will be difficult. Currently available information indicates that at least five dietary components contribute to plasma thiol concentrations and/or redox state. These include the intake of sulfur amino acids, the availability of glutamine, the presence of dietary antioxidants, the content of inducers of glutathione synthesis, and the adequacy of other redox active micronutrients such as selenium, flavin, and niacin (Figure 6).

Animal studies show that steady-state concentrations of tissue GSH, particularly in the liver, vary in association with dietary intake of sulfur amino acids. The total dietary intake for sulfur amino acids (cysteine plus methionine) set by the Food and Agriculture Organization/World Health Organization for adults is 13 mg/kg/day, which is equivalent to about 1 g. The amount of sulfur amino acids present in GSH in the liver is about 1 g of cysteine. Thus, the amount of GSH in the liver is well suited to maintain constant sulfur amino acid supply to support protein synthesis in peripheral tissues despite the temporal nature of sulfur amino acid intake with meals.

Following a meal with sulfur amino acids, tissue supply of amino acids is principally derived from the diet. During this time, increased clearance of Cys from the blood is facilitated by induction of cysteine dioxygenase, which catalyzes the first step in Cys catabolism, and by induction of glutamate-cysteine ligase, which catalyzes the first step in GSH synthesis. The latter is particularly active in the liver so that the liver accumulates a short-term store of Cys. In the postabsorptive state, the release of GSH from the liver provides the major source of Cys, with

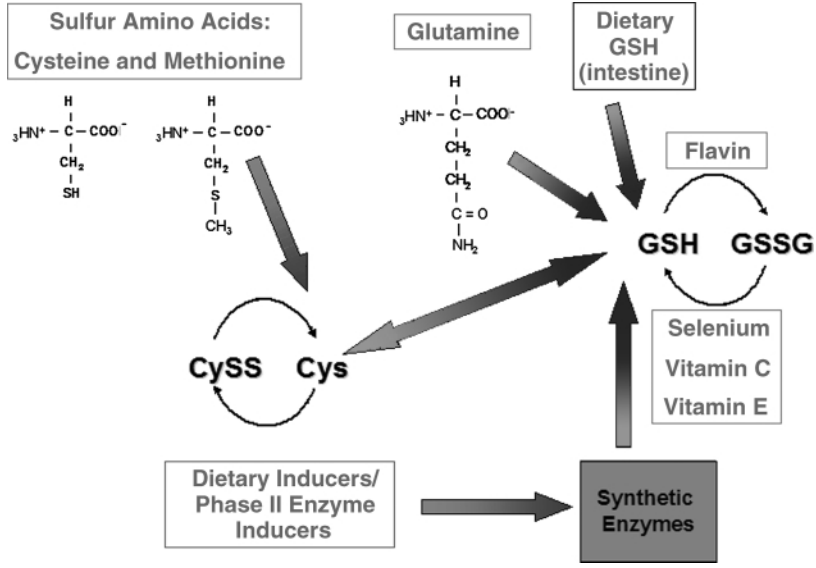

Figure 6 Several dietary components can affect extracellular thiol/disulfide redox state. Direct effects of glutathione (GSH) and glutathione disulfide (GSSG) on redox state in the lumen occur (not shown), but uptake of GSH and GSSG are limited so that direct effects on other pools are not likely. Dietary cysteine and cystine may have direct effects on plasma redox, but under most conditions have an indirect effect due to increased sulfur amino acid pool size derived from both cysteine/cystine and methionine and is associated with a more reduced state. Glutamine (Gln), a precursor for Glu and GSH, is a preferred metabolic fuel for intestine and immune cells. Under some conditions, enhanced Gln supply can increase GSH and promote a more reduced state. A large number of phytochemicals, termed phase II enzyme inducers, function as transcriptional activators to increase expression of detoxification enzymes. These enzymes include glutamate-cystine ligase, the first enzyme in the GSH synthetic pathway. Increased intake of these inducers can, in principle, create a more reduced redox state by increasing steady-state GSH concentration. Dietary antioxidants such as vitamins C and E can inhibit oxidative processes and thereby contribute to a more reduced redox state. Similarly, availability of dietary components needed for the function of the reductase mechanisms, including riboflavin, niacin, and selenium, can affect redox state, at least under deficiency conditions. Cys, cysteine; CySS, cystine.

the relative contribution of this declining as a function of time as the hepatic GSH decreases. During fasting, the contribution of sulfur amino acid supply from protein degradation in other tissues, principally the skeletal muscle, increases. Measurements of whole blood GSH synthesis rate show that these mechanisms maintain nearly constant turnover [fractional synthesis rate was 0.65 ± 0.13 for a normal diet and 0.49 ± 0.13 for a sulfur amino acid restricted diet (80)] despite a substantial difference in sulfur amino acid intake. Because the compensatory mechanisms to maintain sulfur amino acid supply for protein synthesis do so at the expense of capacity for detoxification, optimum sulfur amino acid nutrition cannot be defined in terms of nitrogen balance. In health, this may not be such a critical issue because foreign chemicals in need of detoxification are largely derived from the diet. In contrast, therapeutic exposures often occur under fasting conditions when detoxification functions can be limited by GSH concentration.

In critically ill septic children receiving limited nutritional support, whole blood glutathione synthesis rates were decreased by about 60% (79). In addition, GSH depletion, secondary to inadequate cysteine availability, has been detected in children with various forms of protein energy malnutrition (5, 32, 51, 114). Cysteine supplementation may be critical to improve outcome because increases in GSH levels in children with kwashiorkor are associated with recovery (25). Cysteine supplementation can restore the GSH synthesis rate and thus enhance recovery of the GSH pool (5). Given the normal diurnal variation in GSH associated with sulfur amino acid intake, sulfur amino acids may need to be supplied at regular intervals to maintain detoxification functions during critical illness.

Gln is a nonessential amino acid now recognized to be beneficial under conditions of critical illness (143). Glutamine is a central component in interorgan nitrogen transfer and is also involved in protein and nucleic acid synthesis, gluconeogenesis, and acid-base homeostasis. Glutamine is used as a primary fuel for intestinal mucosal cells, lymphocytes, and other immune cells. Gln is also converted to glutamate, one of the precursor amino acids for GSH synthesis. Extracellular Gln prevents GSH oxidation, and depletion of Gln from culture media causes apoptosis in enterocytes (102, 103). Consequently, support of GSH synthesis and redox homeostasis appear to be included among the critical functions supported by Gln, but additional studies are needed to clarify the mechanisms involved.

Several compounds found in fruits and vegetables activate transcription of glutamate cysteine ligase and increase GSH concentrations in vitro and in animal studies (104). These chemicals, including sulforaphane found in broccoli (24), ellagic acid found in fruits (grapes, pomegranate, strawberries, raspberries) (1), and brassinin found in Chinese cabbage (86, 88), are known as phase II enzyme inducers. These phase II inducers increase detoxification functions and have anticarcinogenic actions (65). Because a high intake of fruits and vegetables is correlated with decreased incidence of chronic disease, better overall health status, and increased plasma antioxidant levels (26, 27, 30), improved thiol status and thiol/disulfide redox state may provide a relevant mechanistic link between diet and health. However, this link remains largely circumstantial under conditions of normal human dietary intake. Health benefits may differ in individuals at risk of

specific cancers or degenerative diseases (140). Because of the increase in use of dietary supplements, additional studies of phase II inducers and their potential benefits and risks in humans are greatly needed.

A number of other well-studied nutrients, including selenium, riboflavin, and niacin, need to be specifically reexamined in terms of their effects on thiol/disulfide redox state. Selenium is required for glutathione peroxidases and TrxR; riboflavin is required for GSSG reductase and TrxR; and niacin is required for the NADPH/$NADP^+$ pool that supports the redox systems controlling thiol/disulfide redox state. True deficiencies of each of these micronutrients are known to have detrimental effects on redox control; however, advances in methodology to more effectively monitor redox state in humans now allow studies to address whether one can optimize nutritional supply in terms of thiol/disulfide redox state. For instance, excess selenium is toxic and causes oxidative stress, whereas adequate selenium is needed to protect against oxidative stress. Thus, direct measurements of extracellular thiol/disulfide redox state may provide a sensitive means to monitor effects of variation in selenium supply, providing a convenient approach to optimize selenium nutrition. In principle, such an approach could provide a useful, central paradigm to define dietary reference intake values.

Summary and Perspectives

Abundant data show that proteins present on cell surfaces and located in extracellular fluids undergo oxidation in diverse pathophysiologic conditions, and accumulating data indicate that the steady-state oxidation is responsive to diet. Methodologies are now available to specifically study redox states of transporters, receptors, and enzymes in response to changes in the extracellular thiol/disulfide redox and to associate these with critical processes such as nutritional absorption, secretory function, neurotransmission, and susceptibility to toxicants. Furthermore, controlled nutritional studies can specifically utilize extracellular redox measurements to explore mechanistic links between diet, health status, and disease. Thus, advances in understanding extracellular thiol/disulfide redox provide the basis for optimizing definition of nutrient requirements and improving nutritional intervention on an individualized basis.

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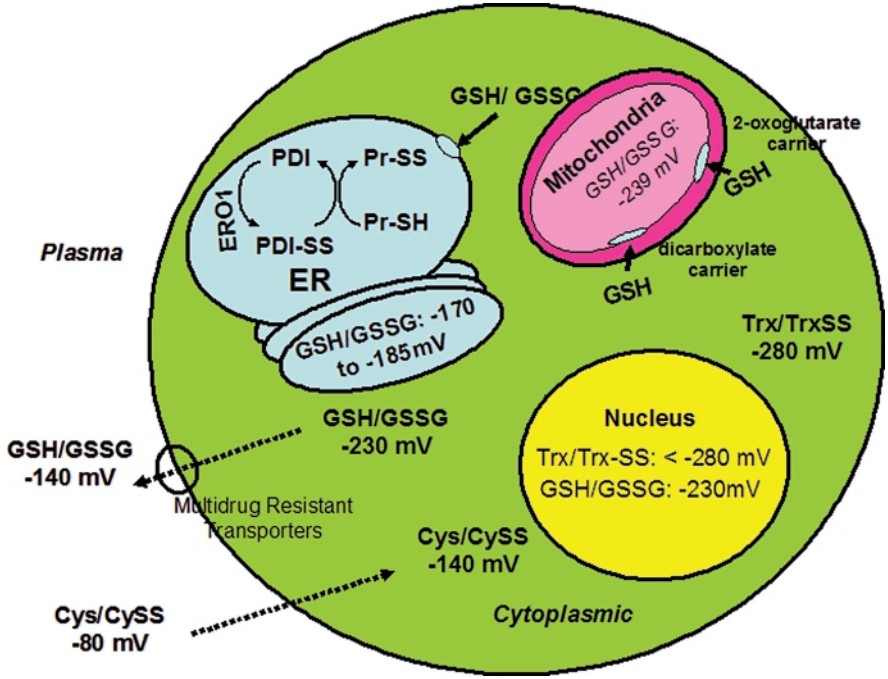

Figure 3 Thiol/disulfide redox states are independently controlled in the cytoplasm (*green*), nuclei (*yellow*), mitochondria (*red*), endoplasmic reticulum (*blue*), and plasma (*white area surrounding cytoplasm*). Most studies on redox compartmentation have addressed glutathione (GSH), the major low-molecular-weight thiol in cells. Analytic difficulties preclude precise measurements for GSH and glutathione disulfide (GSSG) in subcellular compartments, but available data indicate that GSH concentrations in the nucleus and mitochondria are somewhat higher than in the cytoplasm. Initial data on redox state of thioredoxin (Trx) indicate that the nucleus is somewhat more reduced and mitochondria are somewhat more oxidized than cytoplasmic Trx. GSSG is higher in the cisternae of the endoplasmic reticulum, and this results in a considerably more oxidized redox state. GSH is only synthesized in the cytoplasm; known transporters of GSH are shown.

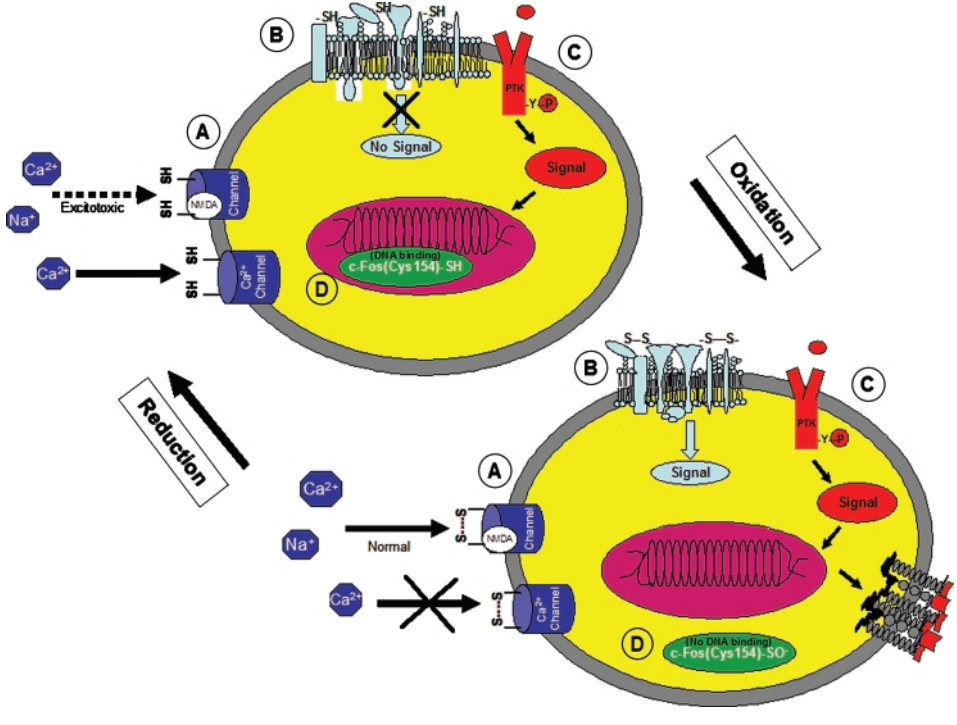

Figure 4 Extracellular thiol/disulfide redox state can affect function of diverse plasma membrane proteins: (*A*) Channels (*dark blue*): Thiol stimulation of the *N*-methyl-D-aspartate (NMDA) receptor can cause excitotoxicity, whereas Ca^{2+} channels can be blocked by thiol oxidation. (*B*) Lipid rafts (*light blue*): Lipid raft clustering can occur through oxidation of protein thiols, leading to downstream signaling. (*C*) Receptors (*red*): Receptor tyrosine kinase signaling can be altered by extracellular redox changes, possibly through extracellular signal-regulated kinases or nuclear factor-B activation, which during oxidizing conditions can lead to extracellular protein expression such as adhesion molecules. (*D*) Transcription factors (*green*): Transcription factor binding can be altered through oxidation of thiols such as in Fos and Jun.

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