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Review

Nutritional biochemistry of cellular glutathione

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Glutathione (GSH) has emerged to be one of the most fascinating endogenous molecules virtually present in all animal cells often in quite high (mM) concentrations. In addition to the detoxicant, antioxidant, and cysteine-reservoir functions of cellular glutathione, the potential of this ubiquitous thiol to modulate cellular signal transduction processes has been recently evident. Lowered tissue GSH levels have been observed in several disease conditions. Restoration of cell GSH levels in a number of these conditions have proven to be beneficial. Thus, strategies to boost cell glutathione level are of marked therapeutic significance. Availability of cysteine, a precursor for glutathione synthesis, inside the cell is a critical determinant of cellular glutathione level. N-acetylcysteine and α-lipoic acid are two pro-glutathione agents that have remarkable clinical potential. The ability of these two clinical drugs to enhance cellular glutathione level, coupled with their favorable effect on the molecular biology of HIV infection may make them useful tools for AIDS treatment. (J. Nutr. Biochem. 8:660–672, 1997) © *Elsevier Science Inc. 1997*

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Introduction

In 1888, de Rey-Pailhade[1,2] observed that yeast cells contain a substance that is responsible for the formation of hydrogen sulfide when the cells are crushed with elemental sulfur. He obtained further evidence that the same substance is present in a number of other tissues e.g., fish and beef muscle, beef liver, fresh sheep blood, sheep brain, egg white, lamb small intestine, and tips of fresh asparagus. In view of its affinity to react with sulfur, the substance was named *philothion* (philo = love, thion = sulfur; in Greek language) by de Rey-Pailhade.[1,2] Hopkins[3] 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 renamed *philothion* as glutathione. Hopkins demonstrated that actually glutathione contained sulfur. In 1929, Hopkins and independently Kendall et al.[4,5] discovered that glutathione was actually a tripeptide and that the peptide contained glycine (Glu-Cys-Gly). Since the discovery of the tripeptide (*Figure 1*), glutathione research has been very popular in all times especially because of the apparent ubiquity and multifaceted functions of the low molecular weight thiol. Much of the current knowledge in glutathione biochemistry has been contributed by Meister and associates who have pioneered the concept of the γ-glutamyl cycle.[6–11] This work provides a concise introduction to glutathione biochemistry and discusses current knowledge of how cellular glutathione may be manipulated by dietary factors. The beneficial potential of proglutathione agents in AIDS therapy has been discussed.

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Functions of glutathione: An overview

Glutathione has emerged to be one of the most fascinating molecules virtually present in all animal cells often in quite high (mM) concentrations. It is known to have multifaceted physiological functions including antioxidant defense, detoxification of electrophilic xenobiotics, modulation of redox regulated signal transduction, storage and transport of

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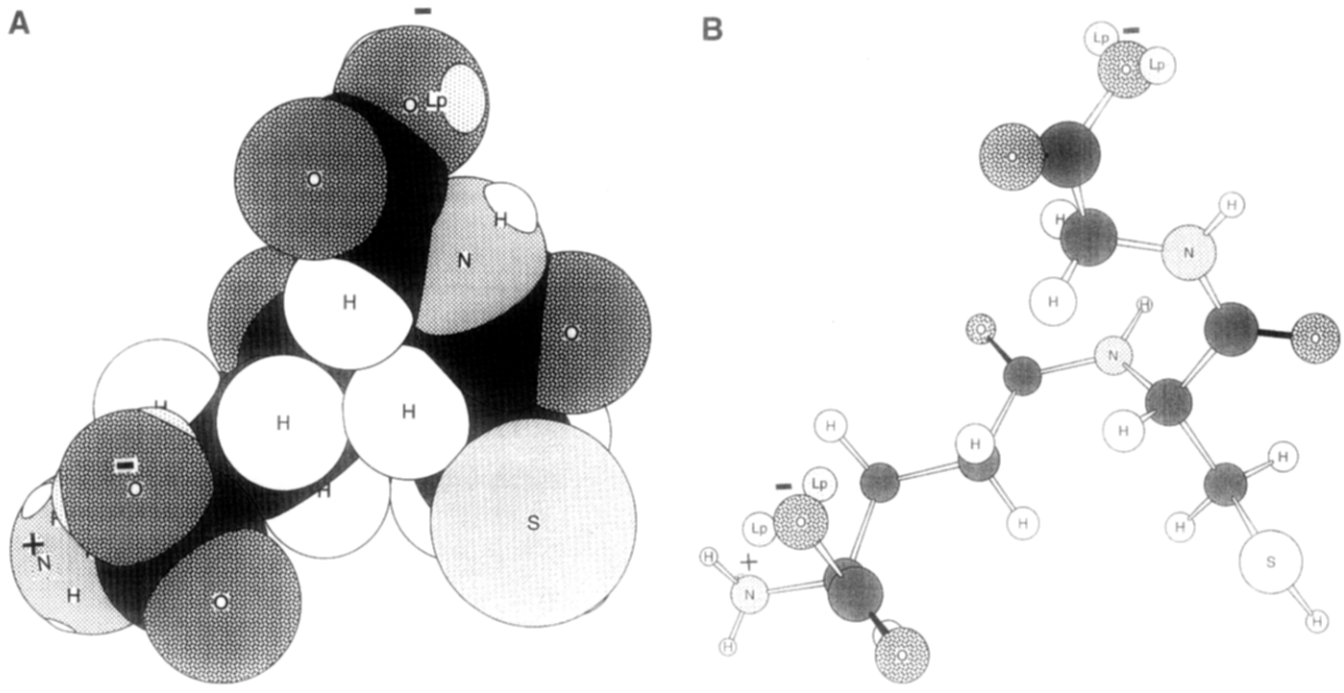

Figure 1 Glutathione. (A) space filling and (B) ball and stick models with element symbols. Under physiological conditions, glutathione is anionic. Lp, lone pair.

cysteine, regulation of cell proliferation, synthesis of deoxyribonucleotide synthesis, regulation of immune response, and regulation of leukotriene and prostaglandin metabolism. A key mechanism that accounts for much of the metabolic and cell regulatory properties of glutathione is thiol-disulfide exchange equilibria. The function of several physiological proteins, including enzymes and signaling molecules, is regulated by thiol-disulfide exchange between protein thiols and low molecular weight disulfides. Thus, the side chain sulfhydryl (-SH) residue in cysteine of glutathione accounts for most of its physiological properties. Disulfide formation is a reversible process that involves a two-electron oxidation. In biology, electron donor and acceptors in this process include molecular oxygen, nicotinamide and flavin cofactors, and other thiols and disulfides. Reversible thiol-disulfide exchange reactions occur by the nucleophilic attack of a thiol on one of the two sulfur atoms of a disulfide (S = S).[12]

Glutathione-related enzymes

Glutathione (L-γ-glutamyl-L-cysteinylglycine) is implicated in the circumvention of cellular oxidative stress and maintenance of intracellular thiol redox status.[11,13–15] Glutathione peroxidase is specific for its hydrogen donor, reduced glutathione, but may use a wide range of substrates extending from H_2O_2 to organic hydroperoxides. The cytosolic and membrane-bound monomer GSH phospholipid hydroperoxide-glutathione peroxidase and the distinct tetramer plasma glutathione peroxidase are able to reduce phospholipid hydroperoxides without the necessity of previous hydrolysis by phospholipase A_2. The protective action of phospholipid hydroperoxide-glutathione peroxidase against membrane damaging lipid peroxidation has been directly demonstrated.[16] Reduced glutathione (GSH) is a major cellular electrophile conjugator as well. Glutathione S-transferases catalyze the reaction between the -SH group of GSH and potential alkylating agents, thereby neutralizing their electrophilic sites and rendering them more water soluble. Glutathione S-transferases represent a major group of phase II detoxification enzymes.[17] Evidence suggests that the level of expression of glutathione S-transferases is a crucial factor in determining the sensitivity of cells to a broad range of toxic chemicals.

Intracellular synthesis of GSH is a tightly regulated two-step process, both of which are ATP dependent. γ-Glutamylcysteine synthetase (also referred to as glutamate-cysteine ligase) catalyses the formation of the dipeptide γ-glutamylcysteine,[18] and subsequently the addition of glycine is catalyzed by glutathione synthetase. Substrates for such synthesis are provided both by direct amino acid transport and by γ-glutamyl transpeptidase (also known as γ-glutamyl transferase) that couple the γ-glutamyl moiety to a suitable amino acid acceptor for transport into the cell. GSH is also generated intracellularly from its oxidized form glutathione disulfide (GSSG) by glutathione disulfide reductase activity in the presence of NADPH. In this context it should be noted that NADH may be transhydrogenated by an energy-dependent mechanism to form NADPH.[19,20] Thus, although glutathione disulfide reductase activity requires NADPH as cofactor, cellular NADH may also contribute to the reduction process. In cells challenged with the oxidant *tert*-butylhydroperoxide almost half of the GSSG formed in the mitochondria was observed to be reduced by NADPH regenerated from NADH.[19]

The activity of γ-glutamylcysteine synthetase, the first step of GSH synthesis, is regulated in vitro by feedback inhibition of GSH itself.[21] This represents an important regulatory mechanism by which the maximum amount of

GSH in the tissues in vivo is limited. Human GSH synthetase is a dimer of two identical subunits, each composed of 474 amino acids. Each subunit contains cysteine residues at positions 294, 409, and 422. Recently it has been shown that Cys-422 is critically important for the activity of the enzyme.[22]

Antioxidant defense network

Apart from functions such as the GSH peroxidase-dependent metabolism of hydroperoxides and direct scavenging of reactive oxygen species, GSH may contribute to antioxidant defense by networking with other major antioxidants such as vitamins E and C.[13,23] GSH plays a critical central role in regenerating vitamins C and E from their oxidized byproducts. In this context it should be noted that the antioxidant activity of selenium and vitamin B_6 is also GSH dependent. Selenium functions as a cofactor of glutathione peroxidase.[24] Vitamin B_6 facilitates the availability of Se for glutathione peroxidase activity.[25]

To obtain best therapeutic results, antioxidant supplementation protocols should consider the requirement of all components of the antioxidant defense network.[26] For example, excess vitamin E in the absence of adequate amounts of regenerating agents will fail to provide full-strength antioxidant protection and accumulation of oxidized vitamin E (α-tocopheroxyl radical) may even lead to the initiation of pathophysiological processes.[27,28] Glutathione and vitamin E appear to be interdependent on each other with respect to the circumvention of oxidative stress-induced cytotoxicity.[29] Oxidized vitamin E radical adhering to low-density lipoproteins may react with polyunsaturated fatty acid containing lipid in the lipoprotein to initiate lipid peroxidation reaction. The potential effects of vitamin E[52] accelerated peroxidation in low-density lipoprotein may be far reaching and profound.[27] Oxidized low-density lipoprotein rich in vitamin E radical was shown to be toxic to endothelial cells. Such exposure was followed by a remarkable loss of intracellular glutathione coupled with release of lactate dehydrogenase and loss of endothelial cell viability. Intracellular glutathione protected against such toxicity.[28] Cytosolic glutathione has been suggested to function as a cofactor for a putative membrane bound vitamin E free radical reductase that regenerates vitamin E from its oxidized byproduct, the tocopheroxyl radical. Pascoe & Reed[29] have reviewed how α-tocopherol may help reduce protein thiyl radicals and prevent the oxidation of such thiols. The relative importance of vitamin E and glutathione in protecting the adult rat brain against ethanol toxicity has been studied.[30] Vitamin E supplementation (oral dl-α-tocopherol, daily dose 10 mg/kg body weight in two divided doses for 15 days) increased glutathione levels in the cerebral cortex and cerebellum. The authors suggested that glutathione-dependent regeneration of the tocopheroxyl radical plays a crucial role in protecting against ethanol-induced damage in the brain. GSH depletion to about 20 to 30% of the total glutathione levels can impair the cell's defense against the toxic actions of reactive oxygen species and may lead to cell injury and death.[31]

In a study performed using healthy, nonsmoking men and women (18 to 50 years of age), vitamin C supplementation (oral, 500 mg/day, 2 weeks) increased mean red blood cell glutathione content by about 50%.[32] It has been shown that ascorbate may spare glutathione consumption by minimizing the glutathione dependent reduction of dehydroascorbate to ascorbate and by providing an alternative cellular reducing agent.[33] It has been explained that perhaps some of the beneficial aspects of vitamin C in health maintenance are related to an interaction between glutathione and vitamin C in vivo.[34,35] Red blood cell and plasma glutathione peroxidase activity (nmol NADPH oxidized/min.ml RBC or nmol NADPH oxidized/min.ml plasma) has been observed to significantly increase in weanling rats supplemented with 2 or 4 g of vitamin C per kg diet for 16 weeks.[36] This suggests that ascorbate may generate such an intracellular environment that facilitates the enzyme-catalyzed hydroperoxide decomposition capacity of glutathione.

Redox regulation of genes

Reactive oxygen species have been associated with the pathogenesis of a large number of human diseases.[37,38] To understand the molecular bases of oxidant and antioxidant action in the aetiology of such disease states current research has been focused on redox-sensitive signal transduction. At least two redox-sensitive transcription factors, nuclear factor κB (NF-κB) and activator protein-1 (AP-1) have been well defined.[39,40] Intracellular thiol redox status seems to be a critical determinant of NF-κB activation. At low levels of cytosolic glutathione disulfide (GSSG), T-cells fail to activate NF-κB in response to appropriate stimuli, whereas high GSSG concentration inhibits the binding of activated NF-κB to its cognate DNA site. Thus, it seems that an intermediate optimal level of intracellular GSSG is required for effective NF-κB activation. Droge et al.[40] have found that glutathione (GSH) deficiency of T-cells is associated with a suppression of NF-κB function. This effect is perhaps related to very low levels of GSSG in GSH-deficient cells. Not much is known about the exact mechanisms underlying ROS-dependent AP-1 activation. Perturbation of cellular thiol redox status has been suggested to be a signal that may be implicated in the induction of c-*fos* and c-*jun* expression caused by asbestos-induced oxidative stress.[41] In support of this, high intracellular glutathione disulfide has been shown to be implicated in AP-1 activation.[42]

Programmed cell death

In several models of programmed cell death or apoptosis, reactive oxygen species have been suggested to be implicated. A characteristic feature in most such models is the loss of intracellular GSH from the apoptotic cells.[43–45] In models such as Fas receptor-mediated apoptosis, intracellular GSH seems to be a key determinant of cell response.[46] Human T cells such as the CEM2DIR are Fas+ but resistant to Fas-mediated apoptosis. CEM2DIR is a variant cell type derived from the Fas-sensitive parental CCRF-CEM cells. Fas+ variants that were resistant to Fas-mediated apoptosis were observed to have higher intracellular GSH content compared with the parental Fas-sensitive cells. When intracellular GSH content of the Fas-resistant variants were decreased by inhibiting cellular GSH synthesis or by grow-

ing cells in a cysteine-deficient medium, Fas resistance was reversed. These observations suggest an important role of GSH in receptor-mediated programmed cell death.[46] In mice thymocytes, GSH has been also shown to inhibit apoptosis triggered by dexamethasone. Treatment of the cells with oxidized glutathione (GSSG), however, induced apoptosis suggesting that the onset of apoptosis is probably regulated by the a change in the ratio of [GSH]i to [GSSG]i.[45]

Downregulation of cell glutathione: Causative factors

The factors that may contribute to decrease tissue GSH are: a) limited GSH synthesis, b) enhanced GSH utilization, and c) limited intracellular reduction of GSSG.

Limited GSH synthesis

As mentioned earlier in this work, GSH synthesis inside the cell is tightly regulated by the level of GSH itself. Therefore, there is a defined ceiling that limits intracellular GSH level. Inborn errors of GSH synthesis are not common. However, deficiencies of γ-glutamylcysteine synthetase as well as GSH synthetase have been reported. Such disorders have been associated with hemolytic anemia and impaired function of the central nervous system. GSH synthetase deficiency has been reported in only about 30 families all over the world. A Japanese boy born in June 1990 was the first Japanese patient reported to suffer from the abnormality.[47] The patient was anemic at birth (Hb 11.9 g/dL) and had a hemolytic attack on postnatal day two. His haemolysis was well compensated, and his second haemolytic episode occurred at 3 years of age. This particular patient has no signs of neurological or mental development impairment, however.[47]

A more common factor that limits intracellular GSH synthesis is the availability of the rate-limiting substrate cysteine. Cysteine, in its reduced form, is highly unstable. Thus, more than 90% of cysteine in human circulation is present in the oxidized cystine form.[48] In cell culture media, this amino acid is only present in the cystine form.

Intracellular GSH synthesis is also subject to hormonal regulation. Hormones that activate cAMP- and Ca^{2+}-dependent cell signalling can downregulate GSH synthesis in the liver.[49–52] Although the mechanism involved in such regulatory processes is yet unclear it has been suggested that calcium-dependent signal transduction pathways could limit the availability of GSH precursor amino acids inside the cell.[49] Also, γ-glutamylcysteine synthetase activity is downregulated by cAMP-dependent protein kinase or protein kinase C-mediated hormone-stimulated phosphorylation of the enzyme.[51] The physiological significance and exact mechanism involved in the hormonal regulation of cellular GSH synthesis is yet unclear. It seems that such hormonal regulation would result in suppressed GSH synthesis in response to increased stress hormone release. This apparently paradoxical response of the cell has been thought to be directed toward preserving cellular cysteine reserves for acute phase protein syntheses in response to stress.[51]

Enhanced GSH utilization

Cellular GSH is highly sensitive to oxidative stress. Thus, exposure to reactive oxygen or nitrogen species result in rapid depletion of cellular GSH. Physiological examples of this is depletion of neutrophil GSH during respiratory burst[53–55] and depletion of muscle GSH reserves during physical exercise-induced oxidative stress.[13,56,57] Oxidation of GSH to GSSG is an early marker of oxidative stress. Rapid blood GSH oxidation in humans performing physical exercise is a typical example of a physiological model.[58,59] Decreased erythrocyte GSH levels in protein energy malnutrition is also a result of increased consumption and not decreased production of GSH.[60] Wasting protein energy malnutrition, kwashiorkor, is predominant in children of developing countries. In affluent countries, many patients suffer from protein energy malnutrition secondary to alcoholism, cancer, and AIDS.

One other major route for GSH utilization is xenobiotic metabolism, such as the metabolism of drugs and pollutants.[17,61–66] Glutathione sulfur transferases catalyze the reaction between the -SH group of GSH and potential alkylating agents, thereby neutralizing their electrophilic sites and rendering them more water soluble and suitable for excretion. Thus, GSH-deficient animals can be prepared by injection of diethylmalate, a substrate for the S-transferase reaction, to experimental animals. GSH deficiency is evident during acetaminophen poisoning and other forms of drug overload.[67–69] Animal studies have shown that extracellular pools of GSH including blood plasma, lung-lining fluid, and small intestinal lumen can be centrally important in protecting against chemical toxicity. GSH in these pools can function to detoxify chemicals extracellularly, supply GSH and its precursors to cells, and protect the extracellular surface of the plasma membrane from damage.[65]

Limited intracellular reduction of GSSG

Reduction of GSSG to GSH is catalyzed by glutathione disulfide reductase present inside the cell. Glutathione disulfide reductase is localized in the cytosol as well as in the mitochondrial matrix. Because of a lack of the presence of this enzyme in the plasma GSSG in the plasma, often contributed by oxidation of GSH in tissues under oxidative stress, cannot be recycled to GSH. Another major consideration is the availability of reducing equivalents for the biological reduction of GSSG. Glutathione disulfide reductase activity requires adequate supply of NADPH as a cofactor. Therefore, conditions that may limit the cellular production of NADPH may be expected to impair reduction of GSSG to GSH resulting in GSSG accumulation in and expulsion from the cell. As a result of such efflux of GSSG, substrates for GSH regeneration in the cell may markedly decrease. Conditions that may decrease cellular NADPH reserves may be exemplified by impaired glucose metabolism and oxidative stress.[19] Inefficient function of the hexose monophosphate shunt will lower NADPH generation. In oxidative stress situations, NADPH is known to be rapidly oxidized. In tissues, oxidative stress is manifested as increased intracellular GSSG/GSH ratio. This is because during the course of its antioxidant function, GSH is

oxidized to GSSG. The rate of GSSG formation exceeds the capacity of the cell to regenerate GSH from GSSG, especially when the availability of NADPH is limited.

Inborn errors of metabolism related to glutathione disulfide reductase are rare but do occur. Such deficiency in glutathione disulfide reductase activity is associated with erythrocyte haemolysis and early cataractogenesis.[70] Inborn deficiency in erythrocyte glucose 6-phosphate dehydrogenase activity impairs NADPH generation and thus increases sensitivity to oxidative stress. These patients suffer from haemolytic anaemia when subjected to oxidative stress.[70]

Downregulation of cell glutathione in health and disease

Tissue glutathione reserves may be downregulated by physiological processes such as aging and acute physical exercise (*Table 1*). Because reactive oxygen species are known to be implicated in aging,[71,72] and produced in excess during exercise[38,56] it is likely that decreased tissue glutathione content is a result of increased utilization of GSH for antioxidant functions. Lowered tissue GSH levels have been also observed in several disease conditions as listed in *Table 1*.

Table 1 Conditions known to be associated with decreased glutathione level

Condition/species	Tissue/cell/subcellular component
HIV+	
human	peripheral blood mononuclear cells[134] lung epithelial lining fluid[161]
Hepatitis C	
human	peripheral blood mononuclear cells[162]
Liver cirrhosis	
human	plasma, erythrocytes[163]
Acetaminophen poisoning	
mice	liver[50]
mice	kidney[69]
Diabetes type II	
human	whole blood[164]
NIDDM	
human	red blood cells[165]
Impaired glucose tolerance	
human	red blood cells[165]
Ulcerative colitis	
human	blood and colonic tissue[166]
Skin burn	
rat and rabbit	skin tissue and mitochondria[167,168]
Idopathetic pulmonary fibrosis	
human	lung epithelial lining fluid[169]
Adult respiratory distress syndrome	
human	lung epithelial lining fluid[170]
Protein-energy malnutrition	
human	erythrocyte[171]
Physical exercise	
rat	liver, muscle and blood[13,26]
Immobilisation atrophy	
rat and dog	skeletal muscles[57,172]
Aging	
mice	liver[173]
human	gastric rnucosa, erythrocytes[163]
rat and mice	mitochondria of liver, kidney and brain[174]
Critically ill	
human	skeletal muscle[175]
Perinatal hypoxia	
human	umbilical arterial cord blood[176]
Acute myocardial infarction	
human	blood[177]
Glutamate cytotoxicity	
human	T cells[116]
rat	neuronal cells[178]
Programmed cell death	
human	T cells[44]

HIV+, human immunodeficiency virus infection; NIDDM, non-insulin-dependent diabetes *mellitus*

Strategies to increase cell glutathione

Tissue GSH synthesis is dependent on dietary amino acid supply. Thus, food-derivation decreases tissue GSH content and refeeding corrects such effect. The sulfur amino acid content of the diet is specifically a major determinant of hepatic GSH content. Dietary cysteine tightly regulates tissue GSH levels within a physiological range.[73–80]

Restoration of cell GSH levels in several pathologies have proved to be beneficial. Strategies to boost cell glutathione level are of marked therapeutic significance. Cell GSH level may be increased by the following ways: a) direct delivery of exogenously synthesized GSH to the cell. and/or b) increased GSH synthesis.

Direct delivery of GSH per se

Administered GSH is not effectively transported into cells[81] except in the small intestine,[82–85] it is mostly degraded in the extracellular compartment. The degradation products, *i.e.*, the constituent amino acids, may be used as substrates for GSH neosynthesis inside the cell. To test the time dependent distribution of intraperitoneally administered GSH, we injected the thiol (1 g/kg body weight) to male Wistar rats.[86] Administration of GSH solution resulted in a rapid appearance ($\uparrow 10^2$ times after 0.5 hr of administration) of glutathione in the plasma. Such response was followed by rapid clearance of plasma total glutathione. After 24 hr of the injection, plasma total glutathione was restored to the pre-injection control level. Excess post-injection 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, and heart. However, after repeated injection of GSH for 3 consecutive days blood and kidney total glutathione levels increased. No such effect was observed in the liver, red gastrocnemius muscle, mixed vastus lateralis muscle, heart, or lung.[86]

Direct elevation of cell GSH reserve is also possible by the administration of GSH esters. Glutathione esters such as GSH mono(glycyl)esters and GSH diethyl esters have been used for this purpose.[81] Unlike GSH itself which is a lipophobic molecule that cannot penetrate the cell membrane, esterified GSH is lipophilic and thus membrane

permeable. However, the use of such esters to boost tissue GSH pool suffer from some limitations. Metal ion contamination of GSH monoesters remarkably decrease the capacity of the compound to serve as a GSH delivering agent. Also, certain forms of esterified GSH such as the GSH dimethyl ester seemed to be toxic to mice.[87] Various monoesters of GSH, e.g., methyl, ethyl, isopropyl, and *n*-propyl have been prepared.[88–90] GSH monoethylester or GSH monoisopropylester seems to be better choices because of the low toxicity of alcohols produced following hydrolysis of the ester.

Some GSH esters are de-esterified by esterase activity in the plasma and lose their ability to permeate the cell membrane. Levy et al.[87] reported that GSH diethyl ester may serve as the GSH delivery agent of choice, especially for those species (e.g., humans but not rat and mouse) that lack GSH diester α-esterase in the plasma.[87] GSH diester was nontoxic to mice and hamsters. Although the idea of using such forms of esterified GSH is very appealing, because of the lack of information from human studies at present, it is premature to consider using esterified GSH for human use.

Increased GSH synthesis

Under normal conditions the rate limiting factor in cellular GSH synthesis is the availability of the constituent amino acid cysteine. Thus, given that the GSH synthesizing enzymes are normal in their activity improving cysteine delivery to cells is often effective to increase cell GSH. Cysteine *per se* is highly unstable in its reduced form. In addition, cysteine is reportedly toxic to cultured cells[91] and to newborn mice[92–94] and rats.[95] As a result, considerable research has been focused on alternative strategies for cysteine delivery.

Certain hormones are capable of increasing GSH content in the liver. The biosynthesis of γ-glutamylcysteine synthetase protein and amino acid transport can be increased in response to insulin and glucocorticoid treatment of cultured hepatocytes. Insulin and hydrocortisone increase GSH synthesis in cultured hepatocytes by inducing the activity of γ-glutamylcysteine synthetase.[52] It has been reported that these hormones may also contribute to cellular GSH synthesis by increasing cystine, a precursor for intracellular cysteine, and glutamate transport.[52,96] Both streptozotocin induced diabetes and adrenalectomy decreases liver GSH reserves of intact animal suggesting that the hormonal regulation of GSH biosynthesis may also occur in vivo.[52,97]

Although, as discussed above, the availability of cysteine within the cell is the most critical limiting step in cellular GSH synthesis, under certain conditions such as severe trauma glutamine supplementation of total parenteral nutrition solutions may promote GSH synthesis. Glutamine supplemented total parenteral nutrition formulations are known to maintain plasma and tissue GSH, and improve survival after acetaminophen toxicity and chemotherapy.[98,99]

Cysteine delivery agents

Several compounds have been tested for their efficacy to serve as cysteine delivery agents. Compounds that have demonstrated potential in humans are discussed below. γ-Glutamyl amino acids inside the cell are substrates of the enzyme γ-glutamyl cyclotransferase, which converts γ-glutamyl amino acids into 5-oxoproline and the corresponding free amino acid. 5-Oxoprolinase catalyses the conversion of 5-oxoproline to glutamate, a substrate for GSH synthesis. This enzyme is found in many tissues except lens and erythrocytes. An analog of 5-oxoproline, L-2-oxothiazalidine-4-carboxylate (OTC), has proven to be a very effective substrate for 5-oxoprolinase.[100–103] As a result of such enzyme OTC reaction, S-carboxy cysteine is thought to be generated. This enzymatic product rapidly hydrolyzes to yield cysteine inside the cell (*Figure 2*).

In humans fed with OTC at 0.15 or 0.45 mmol/kg plasma levels of the drug peaked at about 2h. Although plasma GSH levels were unchanged during the 8 h study, lymphocyte levels of cysteine and GSH markedly increased after 2 to 3 hr.[104] A later clinical trial conducted with HIV-infected patients showed that 6 weeks of OTC treatment (100 mg/kg, 2× per week) resulted in increased level of whole-blood GSH.[105] These important studies underscore the therapeutic potential of OTC in humans.

Two clinically relevant pro-GSH agents that have been most extensively studied so far are *N*-acetyl-L-cysteine (NAC; 2-mercapto-propionyl glycine) and α-lipoate.[23,26,59,106–116] In addition to its reactive oxygen detoxifying properties,[58,107] NAC is thought to function as a cysteine delivery compound.[113,117] After free NAC enters a cell, it is rapidly hydrolyzed to release cysteine. NAC, but not *N*-acetyl-D-cysteine or the oxidized disulfide form of NAC, is deacetylated in several tissues to release cysteine (*Figure 2*). Hydrolysis of oxidized NAC, however, must be preceded by cleavage of the disulfide bridge. NAC appears to be safe for human use because it has been used as a clinical mucolytic agent for many years. Deacetylation of NAC in the intestinal mucosa and possibly in the intestinal lumen is thought to be a major factor determining the low oral bioavailability of NAC.[117] Pharmacokinetics and metabolic studies of NAC show that the oral bioavailability is only around 10%.[109,118–121]

α-Lipoate is also known as thioctic acid, 1,2-dithiolane-3-pentanoic acid, 1,2-dithiolane-3-valeric acid or 6,8-thioctic acid.[23,26,59,115,116] In 1953, Stokstad et al.[122] attempted to study the role of protagon (lipoate) in animal nutrition but failed to produce protagon deficiency in chicks. In 1955, DeBusk and Williams[123] observed appreciable increases in growth rate of chicks and rats fed with diet containing minute quantities of lipoate. Biologically, lipoate exists as lipoamide in at least five 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 lipoamide containing proteins are present in the E2 enzyme dihydrolipoyl acyltransferase, which is different in each of the complexes and 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.[124] Recently lipoic acid has been detected in the form of lipoyllysine in various natural sources. In the plant material

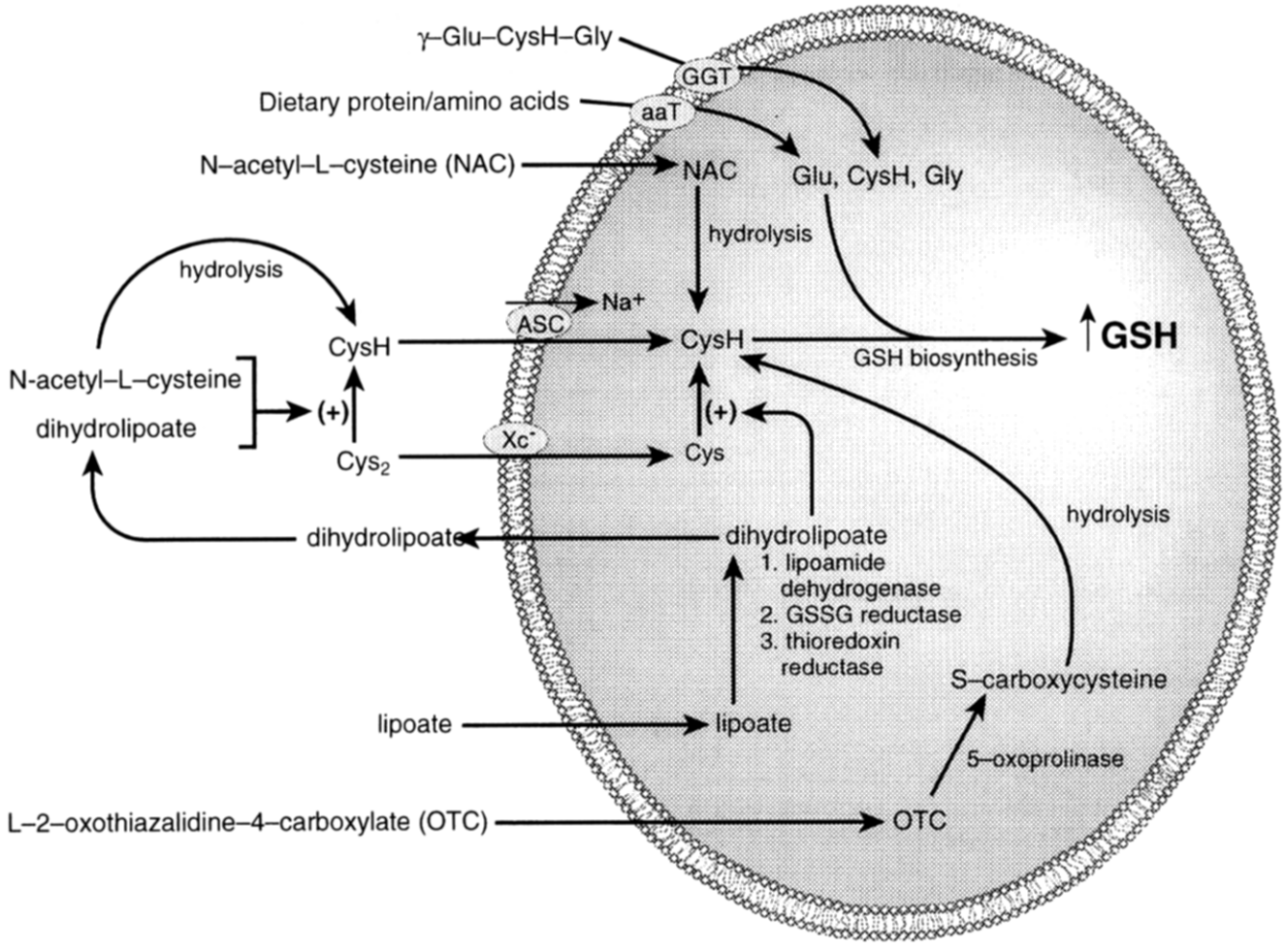

Figure 2 Mechanism of action of the clinically relevant pro-glutathione drugs N-acetylcysteine (NAC), lipoate and L-2-oxothiazalidine-4-carboxylate (OTC). AaT, amino acid transporters; GGT, γ-glutamyltranspeptidase; Cys_2, oxidized cystine disulfide; CysH, reduced cysteine.

studied, lipoyllysine content was highest in spinach (3.15 μg/g dry weight; 92.51 μg/mg protein). When expressed as weight per dry weight of lyophilized vegetables, the abundance of naturally existing lipoate in spinach was over three- and five-fold higher than that in broccoli and tomato, respectively. Lower concentrations of lipoyllysine were also detected in garden pea, brussel sprouts, and rice bran. Lipoyllysine concentration was below detection limits in acetone powders of banana, orange peel, soybean, and horseradish, however. In animal tissues, the abundance of lipoyllysine in bovine acetone powders can be represented in the following order: kidney > heart > liver > spleen > brain > pancreas > lung. The concentration of lipoyllysine in bovine kidney and heart were 2.64 ± 1.23 and 1.51 ± 0.75 μg/g dry weight, respectively.[125]

To develop an understanding of the therapeutic potential of lipoate supplementation, much of the current interest is focused on the fate of exogenously supplemented nonprotein bound lipoate in cell culture, animal and human experimental systems. Lipoate, in its native form, contains a disulfide bond. Reduction of this disulfide results in the conversion of lipoate to the corresponding vicinal dithiol, dihydrolipoate (DHLA). Both lipoate and DHLA have remarkable reactive oxygen detoxifying properties.[23,115]

The mitochondrial E3 enzyme, dihydrolipoyl dehydrogenase reduces lipoate to DHLA in the presence of NADH. The enzyme show a marked preference for the naturally occurring R-enantiomer of lipoate.[126] Lipoate is also a substrate for the NADPH-dependent enzyme glutathione reductase.[127] Glutathione reductase shares a high degree of structural homology with lipoamide dehydrogenase. Both are homodimeric enzymes with 50 kDa subunits conserved between all species. In contrast to dihydrolipoyl dehydrogenase, however, glutathione reductase exhibits a preference for the S-enantiomer of lipoate. Although lipoate is recognized by glutathione reductase as a substrate for reduction, the rate of reduction to DHLA is much slower than that of the natural substrate glutathione disulfide. Whether lipoate would be reduced in a NADH or NADPH-dependent mechanism is largely tissue-specific. Thioredoxin reductase catalyzes the NADPH dependent reduction of oxidized thioredoxin. Thioredoxin reductase from calf thymus and liver, human placenta and rat liver have been observed to efficiently reduce both lipoate and lipoamide in NADPH dependent reactions.[128] Under similar conditions at 20°C and pH 8.0 mammalian thioredoxin reductase reduced lipoic acid 15 times more efficiently than lipoamide dehydrogenase. The relative contribution of the three dif-

ferent enzymes known to reduce lipoate in mammalian cells is tissue- and cell-specific depending on the presence or absence of mitochondrial activity and of oxidized thioredoxin and GSSG.

When lipoate was infused into the liver, thiols were detected in the perfusate.[129] The exact nature of these thiols were, however, not identified. When lipoate was added to murine neuroblastoma and melanoma cell lines, a dose-dependent increase in cellular GSH content was observed in the range of 30 to 70% compared to untreated controls.[130] Consistent results were obtained in an in vivo situation where mice were injected with doses of 4, 8, or 16 mg/kg lipoate for 11 days. Glutathione levels in lung, liver, and kidney of these mice increased significantly.[130] That lipoate can increase cellular glutathione has also been extensively studied in Jurkat T cells as well as human peripheral blood lymphocytes.[116] Lipoate treatment increased cellular glutathione content of Jurkat T cells to 1.5-fold of the control value. We adapted a flow cytometric method to estimate cellular glutathione in lipoate-treated Jurkat T cells and peripheral blood lymphocytes. In Jurkat cells, the effect of lipoate on increasing cellular GSH was observed even at a concentration as low as 10 μM.[116]

Studies with human Jurkat T cells have shown that when added to the culture medium, lipoate readily enters the cell where it is reduced to its dithiol form, DHLA (*Figure 2*). DHLA accumulated in the cell pellet, and when monitored over a 2-hr interval the dithiol was released to the culture medium.[131] As a result of lipoate treatment to the Jurkat T cells and human neonatal fibroblasts, accumulation of DHLA in the culture medium was observed. The redox potential of the lipoate-DHLA couple is −320 mV. Thus, DHLA is a strong reductant capable of chemically reducing GSSG to GSH.

After lipoate supplementation, extracellular DHLA reduces cystine outside the cell to cysteine. The cellular uptake mechanism for cysteine by the ASC system is approximately 10 times faster than that for cystine by the x_c^- system.[132] Thus, DHLA markedly improves cysteine availability within the cell resulting in accelerated GSH synthesis (*Figure 2*).[116,133]

Pro-glutathione agents in AIDS therapy

The potential benefit of pro-glutathione agents in AIDS therapy has been a significant factor contributing to increased research interest for the development of new and more effective strategies to enhance T cell GSH reserves.[59] In 1989, Eck et al.[134,135] were the first to show that human immunodeficiency virus-infected (HIV+) individuals have decreased levels of cysteine and GSH in their plasma and leukocytes. Herzenberg and associates have shown that in HIV+ individuals, T-cell subsets are GSH-deficient.[136–138] Several studies have indicated that restoration of cellular GSH levels by glutathione replenishing drugs may markedly modulate the effect of inflammatory cytokines and inhibit the stimulation of HIV in both acute and chronic infections. Cellular GSH replenishing drugs have proven to be beneficial in this respect.[139–145]

In HIV-infected individuals peripheral blood mononuclear cell GSH levels are decreased.[146] Interestingly, this decrease was mainly confined within T cells. An interesting rend in these observations was that a subpopulation of thiol-rich T cells are most affected.[146] This subpopulation of T cells were decreased or absent in infected patients. In a recent clinical trial with a relatively large HIV-infected subject population, restoration of the thiol status of this subpopulation of cells by supplemental manipulations markedly improved patient survival. In this study, low GSH levels in CD4+ T cells predicted poor survival in AIDS. NAC supplementation to subjects replenished whole blood GSH, and survival of patients who took NAC was higher than would be predicted on the basis on their initial T cell thiol levels.[147] In another clinical trial, double-blind and placebo-controlled, NAC treatment seemed to protect against HIV-infection dependent decline of CD4+ lymphocytes in circulation.[106] Clinical information of the possible effect of lipoate in AIDS therapy is currently not available. However, lipoate has been found to inhibit the replication of HIV-1 in cultured lymphoid T cells.[148]

To evaluate the efficacy of pro-glutathione agents such as NAC and lipoate in AIDS therapy it is essential to consider not only the pro-GSH property of the drugs but also the possible effect of these agents on the molecular biology of HIV infection (*Figure 3*). The long-terminal repeat region of HIV-1 proviral DNA contains two NF-κB binding sites.[149,150] Thus, DNA binding of NF-κB proteins can activate HIV transcription. Strategies to suppress NF-κB-dependent transactivation may be of therapeutic importance to delay the onset and progression of AIDS.[59] NAC is able to suppress NF-κB activation in response to a wide variety of stimuli. Consistently, NAC also inhibited HIV LTR-directed expression of β-galactosidase gene in response to TNFα and phorbol ester.[144,151] However, whether transcriptional regulation of NAC is linked to the pro-GSH effect of NAC is yet unclear. Recently it has been shown that certain transcription dependent effects of NAC such as the enhanced survival of PC12 cells is GSH independent.[152]

Lipoate is also capable of suppressing NF-κB activation in response to a wide variety of stimuli.[39] Treatment of Jurkat T cells with lipoate suppressed phorbol ester or tumor necrosis factor (TNF) α induced activation of NF-κB in a dose-dependent manner.[153,154] This NF-κB inhibitory effect was also seen with DHLA.[155] The ability of lipoate to inhibit NF-κB activation is not dependent on its ability to increase cellular glutathione.[39] Thus, the molecular effects of lipoate are not simply mediated by enhanced cellular glutathione. Enhancing GSH content of lymphocytes of HIV-infected patients may additionally help by specifically suppressing HIV type 1 reverse transcriptase activity.[156] Consistent with the above mentioned findings, lipoate indeed inhibited the replication of HIV-1 in cultured lymphoid T cells.[148]

It is known that high intracellular calcium is involved in oxidant-induced NF-κB activation.[157] Using thapsigargin, an inhibitor of sarcoendoplasmic reticular calcium pumps, cytosolic levels of free calcium can be elevated. Such a condition resulted in oxidant-induced NF-κB activation in Jurkat T cells that are otherwise known to be insensitive to such activation.[157] Independent studies suggest that thapsigargin treatment of T-lymphocyte cells results in marked

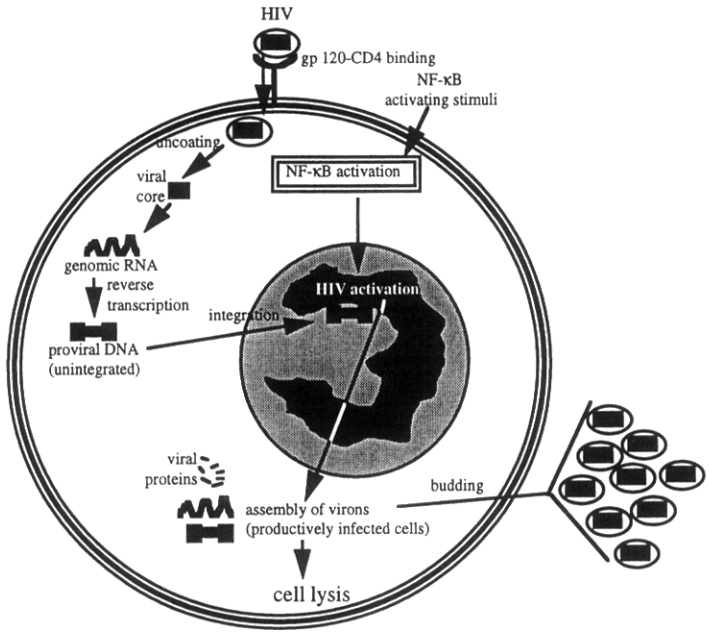

Figure 3 HIV infection of CD4 T cells. Glutathione and pro-glutathione agents have been effective in suppressing reverse transcription of HIV genomic RNA. Also, both N-acetylcysteine and lipoate have been able to effectively suppress NF-κB activity in response to diverse set of stimuli. After HIV infection, glutathione level in T cells have been observed to decrease. Restoration of T cell GSH level under such circumstances seem to be effective in decreasing the loss of CD4 T cells after HIV infection.

activation of HIV production.[158] Viral activation was manifest by increases in soluble viral core p24 production, increases in cellular immunofluorescent staining for viral antigens, and increased viral transcription as measured by HIV LTR-directed expression of the chloramphenicol acetyltransferase reporter gene. This calcium dependent activation of the transcription of proviral HIV may be mediated by NF-κB activation. Pretreatment of cultured T cells with lipoate or NAC diminished oxidant-induced perturbation of intracellular calcium homeostasis.[39,157] Such effect of the drugs on cell calcium metabolism may account for some of the beneficial properties of both lipoate and NAC against HIV infection.[59]

HIV-infected patients have elevated plasma glutamate levels.[159] As discussed in a previous section, availability of cysteine inside the cell is the rate limiting step in cellular glutathione synthesis. Because of its marked instability in the reduced form more than 90% of extracellular cysteine is present as cystine.[48] Glutamate competitively inhibits x_c^- system-dependent cystine uptake by cells.[132] In this way, substrate for glutathione synthesis within the cell is limited in the presence of high concentrations of extracellular glutamate. In Jurkat T cells we have observed that supplementation of 5 mM glutamate to the culture medium results in 50% decrease of cellular GSH level.[116] Our flow cytometric studies revealed that lipoate treatment can increase cellular GSH levels on a dose-dependent manner from 10 to 100 μM. A very interesting observation was that lipoate can bypass glutamate inhibition of glutathione synthesis in human lymphocytes. Based on results obtained in our laboratory we have developed the following mechanistic explanation. When treated to cells, lipoate rapidly enters the cells and is reduced to DHLA. DHLA expelled from the cell to the culture medium reduces extracellular cystine to cysteine. Cysteine, taken up by the ASC system, can bypass glutamate inhibition of cystine uptake and can provide sufficient substrate for cellular GSH synthesis (*Figure 2*). Because these effects of lipoate are observed at concentrations 100 μM or below, they should be considered to be clinically relevant.[116]

Among the clinically relevant pro-glutathione agents tested so far undoubtedly NAC and lipoate hold most promise. Although in many respects the effect of NAC are quite similar to that of lipoate, much higher concentrations of NAC are required to produce comparable effects. For example, under experimental conditions 10–30 mM NAC is used to obtain inducible NF-κB inhibition. In contrast, 1 mM lipoate is clearly effective in suppressing NF-κB activation in response to a large number of stimuli. In Jurkat T cells although 100 μM NAC failed to enhance cellular GSH level, lipoate could do so in a dose dependent manner from 10 to 100 μM as observed in our flow cytometric assays.[116] A recent HIV related study directly compared the efficacy of NAC and lipoate with respect to NF-κB-mediated gene expression.[160] At 200 μM, lipoate treatment resulted in a 40% decrease in HIV-1 p24 antigen expression in TNFα stimulated OM 10.1 cells latently transfected with HIV1. In contrast, 10 mM NAC was required to produce comparable effects. Much of these concentration differences may be explained by the mechanism of lipoate action. An unique advantage of lipoate is that it is able to utilize cellular reducing equivalents, and thus harnesses the metabolic power of the cell, to continuously regenerate its potent dithiol form. Because of such a recycling mechanism, the lipoate-dihydrolipoate couple can be continuously maintained in a favorable redox state at the expense of the cell's metabolic power. This is not possible for NAC, and thus higher concentrations are necessary to obtain comparable effects.

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