invited review

Lung glutathione and oxidative stress: implications in cigarette smoke-induced airway disease

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Rahman, Irfan, and William MacNee. Lung glutathione and oxidative stress: implications in cigarette smoke-induced airway disease. *Am. J. Physiol.* 277 (*Lung Cell. Mol. Physiol.* 21): L1067–L1088, 1999.—Glutathione (GSH), a ubiquitous tripeptide thiol, is a vital intra- and extracellular protective antioxidant in the lungs. The rate-limiting enzyme in GSH synthesis is γ-glutamylcysteine synthetase (γ-GCS). The promoter (5′-flanking) region of the human γ-GCS heavy and light subunits are regulated by activator protein-1 and antioxidant response elements. Both GSH and γ-*GCS* expression are modulated by oxidants, phenolic antioxidants, and inflammatory and anti-inflammatory agents in lung cells. γ-GCS is regulated at both the transcriptional and posttranscriptional levels. GSH plays a key role in maintaining oxidant-induced lung epithelial cell function and also in the control of proinflammatory processes. Alterations in alveolar and lung GSH metabolism are widely recognized as a central feature of many inflammatory lung diseases including chronic obstructive pulmonary disease (COPD). Cigarette smoking, the major factor in the pathogenesis of COPD, increases GSH in the lung epithelial lining fluid of chronic smokers, whereas in acute smoking, the levels are depleted. These changes in GSH may result from altered gene expression of γ-*GCS* in the lungs. The mechanism of regulation of GSH in the epithelial lining fluid in the lungs of smokers and patients with COPD is not known. Knowledge of the mechanisms of GSH regulation in the lungs could lead to the development of novel therapies based on the pharmacological or genetic manipulation of the production of this important antioxidant in lung inflammation and injury. This review outlines *1*) the regulation of cellular GSH levels and γ-*GCS* expression under oxidative stress and *2*) the evidence for lung oxidant stress and the potential role of GSH in the pathogenesis of COPD.

γ-glutamylcysteine synthetase; oxidants; antioxidants; activator protein-1; antioxidant response element; smokers; lungs; chronic obstructive pulmonary disease; airway epithelium

GLUTATHIONE (GSH) is a ubiquitous, essential tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) containing a sulfhydryl group that enables it to protect cells against oxidants, electrophilic compounds, and xenobiotics. GSH, which accounts for 90% of intracellular nonprotein thiols, is a key intracellular reducing agent and is implicated in immune modulation and inflammatory conditions (155, 157). GSH also serves as a storage and transport form of cysteine and as a cofactor in several enzymatic reactions. Hence GSH is emerging as one of the fundamental antioxidant defense mechanisms in oxidant-induced lung injury and inflammation. Alterations in lung lining fluid GSH levels have been shown in various inflammatory conditions. For example, it is decreased in the epithelial lining fluid (ELF) of idiopathic pulmonary fibrosis (IPF) (40, 146), acute respiratory distress syndrome (35), cystic fibrosis (217), and human immunodeficiency virus-positive (235) patients.

Chronic obstructive pulmonary disease (COPD) is a condition characterized by progressive and largely irreversible airway obstruction and an influx of inflammatory cells into the lungs (201, 206, 232). Mortality and morbidity from the disease are high in developed countries and are rising in developing countries (232). The important events in the pathogenesis of COPD are considered to be lung inflammation, an increased oxidant burden, and a protease-antiprotease imbalance in the lungs (202, 205, 206, 232). The increased oxidant burden derives from the fact that cigarette smoke contains an estimated 10^{14} free radicals/puff and that many of these, such as tar semiquinone, which can generate H_2O_2 by the Fenton reaction, are relatively

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long-lived (178, 194, 268). It is reported that >90% of patients with COPD are smokers, but not all smokers develop COPD (232). Fifteen to twenty percent of cigarette smokers appear to be susceptible to its effects, show a rapid decline in forced expiratory volume in 1 s (FEV_1), and develop the disease (232). The reasons for this are not clear but may involve genetic predisposition, dietary habits, differences in depth or pattern of inhalation, variations in cellular and biochemical responses, and differences in immune or regenerative capacity of lung cells. Epidemiological evidence leaves no reasonable doubt that cigarette smoke is the major causative agent of COPD, with atmospheric pollution as an additional contributory factor. Studies carried out with an animal model and an alveolar epithelial cell line (A549) in vitro showed that the thiol antioxidant GSH is critical to lung cellular antioxidant defenses, particularly in protection from oxidant injury (129, 131). GSH is present in increased concentrations in the ELF of chronic smokers, whereas this does not occur in the ELF of acute smokers (42, 166). There is a large gap in our understanding of the metabolism of GSH in both the various anatomic compartments and the cell types within the lung. In addition, information is lacking on GSH levels and GSH regulation in the lungs of smokers and patients with COPD.

Oxidant-sensitive transcription factors such as activator protein-1 (AP-1), which consists mainly of c-Fos and c-Jun homo- or heterodimers are known to play a key role in proinflammatory processes such as the transcription of cytokine genes and also in upregulating protective antioxidant genes (196). Recent evidence (204) suggested that oxidants, phenolic antioxidants, and inflammatory and anti-inflammatory agents modulate the activities of AP-1. AP-1 has also been reported to modulate the expression of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in de novo GSH synthesis. γ-GCS consists of a catalytic heavy subunit (γ-GCS-HS) and a regulatory light subunit (γ-GCS-LS). It has recently been shown that the promoter (5′-flanking) regions of the human catalytic *γ-GCS-HS* and regulatory subunit *γ-GCS-LS* genes contain a putative AP-1 and an antioxidant response element (ARE) that are necessary for *γ-GCS* expression in response to diverse stimuli (81, 199, 203). It is possible that differences in ELF GSH in various inflammatory lung diseases are due to changes in the molecular regulation of GSH synthesis in lung cells by AP-1 and ARE. There are excellent reviews available describing aspects of the antioxidant GSH (58, 83), oxidant-induced lung injury (26, 202, 212) and toxicity (208, 230), and the protective role of antioxidants (86, 252). The primary objective of this review is to present a detailed account of the current knowledge of the regulation of lung GSH and γ-GCS in conditions of oxidative stress in smokers and patients with airway diseases such as COPD. Second, this review explores the molecular mechanisms by which this antioxidant molecule is modulated in oxidant-mediated lung injury and inflammation.

BIOSYNTHESIS OF GSH

The synthesis of GSH requires the presence of two enzymes; ATP; Mg^{2+}; and the amino acids glycine, cysteine, and glutamate, with cysteine being the rate-limiting substrate. The tripeptide GSH is formed by the consecutive actions of γ-GCS and GSH synthetase (Fig. 1) (157). Both enzymes are exclusively cytosolic, and the rate of GSH synthesis is controlled by the amount of γ-GCS present, the availability of L-cysteine, and feedback inhibition excerted by GSH on γ-GCS (214).

GSH synthetase apparently has no regulatory role; once synthesized, γ-glutamylcysteine is rapidly converted to GSH. The activity of γ-GCS determines the rate of GSH synthesis. γ-GCS-HS contains binding sites for all three substrates and all essential catalytic residues. The mammalian γ-GCS holoenzyme is a heterodimer consisting of a 73-kDa γ-GCS-HS and a 30-kDa γ-GCS-LS (223). Although the HS contains all of the catalytic activity, HS activity can be modulated by the association with the regulatory γ-GCS-LS (100). It has been calculated that 80% of the cytosolic γ-GCS protein is inactive under physiological conditions due to

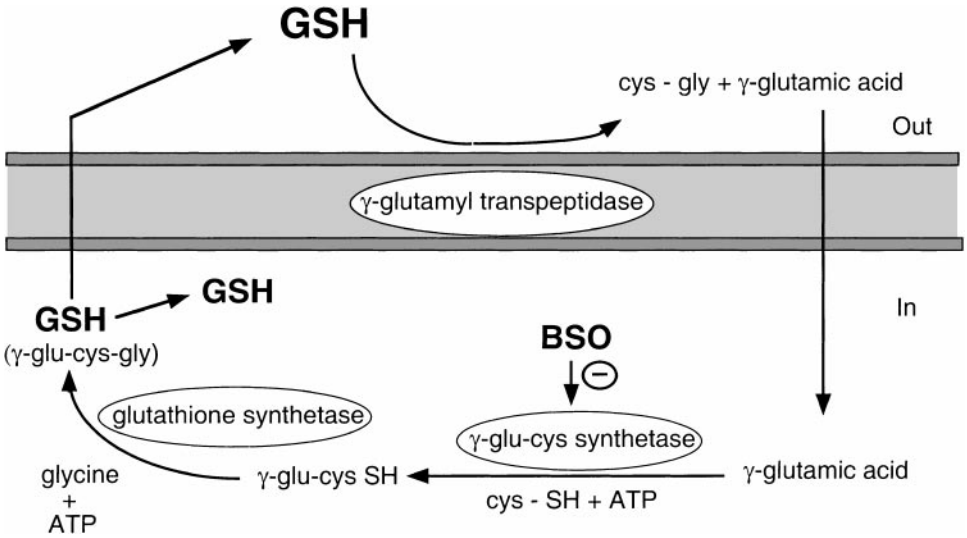

Fig. 1. Steps in de novo glutathione (GSH) biosynthesis and degradation of extracellular GSH in lung cells. DL-Buthionine-(*SR*)-sulfoximine (BSO) is an inhibitor of γ-glutamylcysteine synthetase (γ-GCS) enzyme. γ-Glutamyl transpeptidase, γ-GCS, and glutathione synthetase enzymes are discussed in text. SH, sulfhydryl group.

binding with GSH (100). Thus a decrease in GSH triggers the release of the GSH bound to γ-GCS, which, in turn, results in increased levels of active γ-GCS and hence enhanced synthesis of GSH. This process does not require de novo synthesis of γ-GCS protein and is one way by which cells control their GSH levels when challenged by agents that lead to an initial depletion of intracellular GSH.

The regulatory properties of γ-GCS-LS are proposed to be mediated by a disulfide bridge between the subunits that would allow conformational changes in the active site depending on the oxidative state of the cell (100). An important cysteine residue has been identified in the active site of γ-GCS-HS by site-directed mutagenesis, which is involved in heterodimer formation between γ-GCS-HS and γ-GCS-LS (247). This suggests that the potential for increasing the rate of GSH synthesis exists under conditions of GSH depletion.

GSH AND ITS REDOX SYSTEM

The GSH redox system is crucial in maintaining intracellular GSH homeostasis, which is critical to normal cellular physiological processes and represents one of the most important antioxidant defense systems in the lung (39). This system uses GSH as a substrate in the detoxification of peroxides such as hydrogen peroxide (H_2O_2) and lipid peroxides, a reaction that involves glutathione peroxidase (GPx). This reaction generates oxidized GSH (GSSG), which is subsequently reduced by glutathione reductase in a reaction that requires the hexose monophosphate shunt pathway utilizing NADPH (Fig. 2). Physiologically, the glutathione reductase reaction is driven strongly in favor of GSH, with the GSH-to-GSSG ratio normally >90%. Maintenance of the high GSH-to-GSSG ratio minimizes intracellular accumulation of disulfides. However, if oxidant stress or other stress alters this ratio, the consequent shift in the GSH-to-GSSG redox buffer influences a variety of cellular processes such as activation of the transcription factors AP-1 and nuclear factor-κB (NF-κB). The protective functions of GSH involve enzymatic as well as nonenzymatic processes. GSH is a strong nucleophile and often inactivates electrophilic reactive compounds either by nonenzymatic direct conjugation or by an enzyme-catalyzed reaction involving glutathione *S*-transferase (GST).

COMPOSITION OF GSH IN LUNGS

The lung is constantly exposed to many atmospheric pollutants such as cigarette smoke, ozone, and nitrogen dioxide and is also at risk from oxidant injury by inhalation of high concentrations of oxygen. It contains the largest endothelial surface area of any organ, which makes the lung a major target site for circulating oxidants and xenobiotics. It is therefore no surprise that the human lung is one of the important storage areas for GSH (6.1–17.5 nmol/mg lung) (19, 49). Lung extracellular ELF is rich in the antioxidant GSH, which detoxifies oxidants, free radicals, organic polyaromatic hydrocarbons, and electrophilic compounds (200, 208). Thus extracellular GSH in the lungs can protect alveolar macrophages, pulmonary epithelial cells, and pulmonary endothelial cells from oxidative stresses and helps to maintain functional surfactant (52, 93, 226, 245). GSH concentrations vary throughout the respiratory tract, being lower in nasal lining fluid than in alveolar lining fluid (52). GSH levels in the ELF (200–400 μM) of the lungs are ~100 times higher than those in plasma (2–4 μM) (52). The half-life of cytosolic GSH in the lungs is not known, but its half-life is 0.5 and 3 h in kidney and liver cells, respectively, compared with that in human plasma where its half-life is <2 min (259).

ROLE OF γ-GLUTAMYL TRANSPEPTIDASE IN THE REGULATION OF GSH LEVELS IN LUNGS

Intact GSH is not taken up at a significant rate by the lungs. Extracellular GSH is broken down into its constituent amino acids by γ-glutamyl transpeptidase (γ-GT) and is resynthesized intracellularly rather than by direct cellular uptake (1, 89). The enzyme γ-GT is a plasma membrane enzyme, with its active site directed toward the outside of the cell, present in lung epithelial cells. This enzyme breaks the γ-glutamyl bond of γ-glutamyl-cysteinyl-glycine (Fig. 1) (218). The glutamyl moiety is then transferred to an amino acid, a dipeptide, or GSH itself, producing its γ-glutamyl derivative. Thus γ-GT acts as a salvage enzyme for cellular GSH synthesis (72). The lung epithelium has been shown to have high levels of γ-GT activity and utilizes extracellular GSH from the alveolar lining fluid (17). Hence most of the plasma GSH is catabolized by the enzyme γ-GT in lungs (39, 90). As a result, γ-GT may be important in determining the levels of GSH in lung ELF. Endothelial cells, alveolar macrophages, and fibroblasts have lower γ-GT levels and therefore less easily use extracellular GSH for intracellular GSH synthesis (17, 218).

In an animal model, rats exposed to hyperoxia exhibited low γ-GT activity in ELF, which was associated with low ELF GSH levels (252, 254). γ-*GT* expression is increased in rat lung epithelial cells by oxidants such as menadione and *t*-butylhydroquinone (122), suggesting

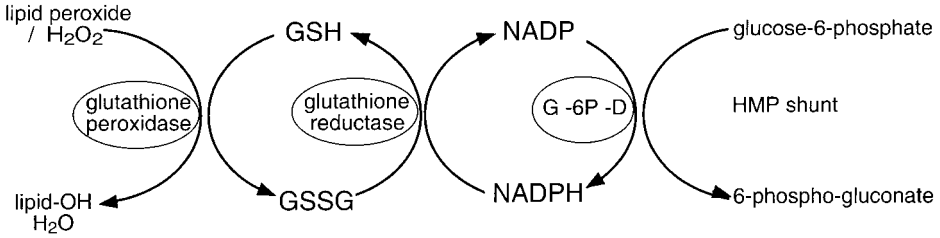

Fig. 2. GSH redox cycle. GSH converts hydrogen and lipid peroxides to nontoxic hydroxy fatty acids and/or water. Glutathione disulfide (GSSG) is subsequently reduced to GSH in presence of NADPH and glutathione reductase, which are linked with hexose monophosphate (HMP) shunt. G-6-PD, glucose-6-phosphate dehydrogenase.

that γ-GT might play a role in the protection against oxidative stress. However, cigarette smoke condensate and oxidative stress had no effect on γ-GT activity in human type II alveolar epithelial cells (A549 cells) (197). The possible explanation for the differential regulation of γ-GT activity in response to oxidants may be due to differential expression of the γ-*GT* gene in different cell lines and organs and in different species. Furthermore, the direct involvement of γ-GT in the regulation of GSH levels in the lungs of smokers remains unproven.

REGULATION OF ELF GSH

Lung ELF GSH may come from a variety of sources. Simple diffusion from the plasma is unlikely because blood levels of GSH are 100 times lower than those in ELF, with values on the order of 0.5–5 μM (52, 259). It is likely that GSH is transported out of cells; intracellular GSH levels are regulated in part by the rate of such a membrane bidirectional transport system as in lung and liver cells (142, 253). The function of such a GSH transport system is influenced by the redox or thiol status of the cell, the membrane potential, and the presence of cations in the extracellular environment (102, 141). GSH-related structural compounds, such as glutathione S-conjugates and GSH ethyl ester (GEE), inhibit cellular GSH uptake or influx (102, 253). Furthermore, a more oxidized extracellular environment stimulates cells to retain GSH, whereas a more reduced extracellular state facilitates GSH efflux (138, 253). However, these effects are in direct contrast with the situation in vivo in the lungs because the increased oxidant burden imposed by smoking and endogenous oxidative stress should cause lung cells to retain rather than release GSH into the ELF. This mechanism is difficult to explain by the presence of such a bidirectional GSH transporter in the lung. Thus the mechanisms that determine the levels of GSH in lung ELF are not fully understood.

REGULATION OF CYSTINE TRANSPORT AND GSH LEVELS IN LUNG CELLS: EFFECTS OF OXIDATIVE STRESS

The rate-limiting step in the biosynthesis of GSH is the availability of cysteine as a substrate within the cell (157). Cystine, an oxidized form of cysteine, is efficiently transported into cells by the specific inducible Na^+-independent anionic amino acid transport x_c^- mechanism and subsequently reduced for use in various metabolic processes including GSH synthesis in lungs (13, 58, 63). Intracellular transport of cystine is accompanied by the extracellular release of glutamate. Cysteine is also transported into cells by Na^+-dependent pathways (A or ASC) shared with glutamine and serine (11). It has been reported that isolated rat alveolar type II cells have a constitutive noninducible Na^+-dependent active uptake system that transports exogenous GSH and its γ-glutamyl analogs into the cells against a concentration gradient (10, 34, 93). These transport systems may act to increase intracellular GSH in lung cells.

Various forms of oxidant stress and nitric oxide (NO) also increase the activity of membrane cystine and glutamate transport, leading to increased GSH synthesis in lung cells (57, 63, 127). It has been clearly shown that cystine uptake is the rate-limiting step for GSH synthesis in cultured lung cells, especially under conditions of oxidative stress (12, 62). Glutamate or glycine is rarely rate limiting. Oxidants (hyperoxia and H_2O_2) and agents such as sodium arsenite, cadmium, electrophilic compounds, and diethyl maleate also induce cystine transport in various lung cells, macrophages, and erythrocytes that is analogous to the x_c^- transport system, a Na^+-independent inducible system specific for intracellular transport of cystine and glutamate (56, 57, 188, 237). Deneke and colleagues (59, 61) have shown that exposure of rats to hyperoxia resulted in increases in total lung GSH within 24 h. It is therefore possible that the induction of cystine or cysteine transport could contribute to the increased GSH levels in the lungs after exposure to hyperoxia (59, 61).

The regulation of cystine-glutamate transport is governed by the availability of extracellular cysteine or cystine as well as by the extracellular redox state (which is, in part, determined by extracellular GSH levels) (13, 189). Treatment with reducing agents such as *N*-acetyl-L-cysteine (NAC) or GSH increases intracellular GSH levels by reducing cystine to cysteine in bovine pulmonary artery endothelial cells (58). Furthermore, NAC increases intracellular GSH levels in bovine pulmonary artery endothelial cells even in the absence of cystine in the medium, possibly not mediated by mixed disulfide formation (189). This suggests that a different transport mechanism independent of the x_c^- system may be involved in type II epithelial cells to increase GSH levels in response to various stresses (34). This is one of the mechanisms whereby lung cells increase intracellular GSH levels under various stresses (either oxidant stress or GSH depletion).

MOLECULAR REGULATION OF GSH SYNTHESIS IN LUNG CELLS

Transcriptional Level

The liver and lungs are the major sources of GSH metabolism and have higher levels of γ-GCS than other tissues (39, 87, 157). Studies have been performed on alveolar epithelial type II cells to elucidate the potential role of these cells in the regulation of cellular GSH turnover in the lung (252). Alveolar epithelial type II cells are more metabolically active than other lung cells (51, 64) and represent a relatively small proportion (4–5%) of the total air space cell population (79). The molecular mechanisms of GSH synthesis and regulation in type II alveolar epithelial cells in response to various environmental, oxidant, and inflammatory stimuli have been studied. We (203) and other investigators (238, 243) have recently reported that the promoter (5′-flanking) region of the human γ-*GCS-HS* gene is regulated by a putative c-Jun homodimeric complex-AP-1 sequence. This sequence is located at the

proximal region of the γ-GCS-HS TATA box in various cell lines including human alveolar epithelial cells (203, 238, 243). Monova and Mulcahy (161) and Mulcahy et al. (170), however, have reported an ARE containing an embedded phorbol 12-myristate 13-acetate response element (TRE/AP-1) and an electrophile response element (EpRE; or its functional equivalent, ARE), which play a key role in the regulation of γ-*GCS-HS* and γ-*GCS-LS*, respectively, in response to a planar aromatic xenobiotic compound, the phenolic antioxidant β-naphthoflavonone, specifically in a hepatoma cell line (HepG2 cells). They also showed that the internal AP-1 site is important for the constitutive expression of the γ-*GCS-LS* gene (161). However, recently, Galloway et al. (82) were unable to show a role for ARE in the induction of γ-*GCS-LS* by oxidants such as *t*-butylhydroquinone in HepG2 cells. They suggested that an AP-1 site was the critical element for the constitutive regulation of this subunit.

A role for NF-κB in the modulation of γ-*GCS-HS* gene expression has also been suggested (103, 250). It has been shown that blocking the activation of NF-κB that is present at the transcriptional site of the γ-GCS-HS promoter by various strategies prevented the oxidant- or cytokine-induced increase in γ-*GCS-HS* transcription in mouse endothelial cells and hepatocytes (36, 250). However, mutation and deletion strategies in the γ-GCS-HS promoter region have ruled out the possible involvement of NF-κB in the transcriptional upregulation of the γ-*GCS-HS* gene in alveolar epithelial cells and other cell lines in response to tumor necrosis factor (TNF)-α and oxidative stress (164, 196, 198, 203, 224). In addition, the role of the metal response element-binding transcription factor-1 (MTF-1), which is present in the promoter region of γ-*GCS-HS*, has been suggested in the transcriptional control of γ-*GCS-HS* gene expression in response to heavy metals (92). The transcription of γ-*GCS-HS* mRNA is largely diminished in the livers of MTF-1-null mice, establishing a potential link between the MTF-1 in the regulation of GSH biosynthesis and protection from metal-induced oxidative stress. Therefore, it is likely that the expression of the γ-*GCS* genes is regulated distinctly in a variety of cells at the transcriptional level by different regulatory signals in response to diverse stimuli.

Translational Level

Modulation of GSH synthesis has also been described at the posttranslational levels in the rat liver in vivo (15). Various inflammatory agents such as cAMP and intracellular calcium that are released during inflammation may inhibit GSH synthesis at the translational level (140). It has been shown that γ-GCS activity is inhibited by agonists of various signal transduction pathways in rat hepatocytes (140), suggesting a role for signaling mechanisms in the regulation of GSH levels. Lu et al. (140) reported that hepatic GSH synthesis is downregulated in response to hormones known to mediate their effects through the activation of distinct signal transduction pathways. Using various specific inhibitors of signaling pathways, these investigators determined that the hormone-specific inhibition of GSH synthesis was mediated by the activation of protein kinase A, protein kinase C, and Ca^{2+}/calmodulin-dependent kinase II. This inhibition of GSH synthesis was correlated with the direct phosphorylation of γ-GCS-HS on serine and threonine residues in a Mg^{2+} concentration-dependent fashion. Phosphorylation of γ-GCS-HS was also detected in rat hepatocytes treated with dibutyryl cAMP, resulting in the inhibition of γ-GCS activity in vivo (236). Thus phosphorylation-dephosphorylation may regulate γ-GCS activity (236) and may provide a mechanism for altering GSH levels in lung cells during oxidative stress.

OXIDATIVE STRESS: INTRACELLULAR GSH AND γ-GCS REGULATION IN LUNG CELLS AND CELLULAR TOLERANCE

As a result of various oxidative stresses, GSH may form a protein-mixed disulfide with sulfhydryl (-SH) group-rich protein moieties such as albumin (52, 157). GSH also undergoes oxidation to form GSSG and a thiyl group, which are toxic to the cells (83, 157, 208). The relationship between decreased GSH content, increased formation of GSSG or protein-mixed disulfide, and increased cellular sensitivity to a variety of agents that impose oxidative stress is well established (83).

Oxidative stress may initially deplete GSH, followed by an increase in intracellular GSH levels, as a result of induction of γ-GCS-HS (197–199). Rapid depletion of intracellular GSH has been shown to occur with exposure to cigarette smoke or its condensate in epithelial cells in vitro and in rat lungs in vivo (129, 200). This is followed by a later rebound increase in GSH in epithelial cells as an adaptive response to oxidative stress, which occurs as a result of upregulation of γ-*GCS-HS* and activation of AP-1 (199). This adaptive response may explain the increase in GSH in ELF in chronic smokers (42, 166). In addition, after the initial depletion of GSH by oxidants such as H_2O_2, redox recycling, menadione, and hyperoxia, there is also a later increase in GSH at 12–24 h in lungs in vivo and in human alveolar and bronchial epithelial and endothelial cells in vitro (96, 108, 191). This is associated with an increased expression of mRNA for the γ-*GCS* subunit genes. Thus oxidants appear to upregulate the gene for GSH synthesis (Table 1). This presumably acts as a protective mechanism against oxidative stress. Table 1 categorizes the main inducers of γ-*GCS-HS* and γ-*GCS-LS* in lung cells. However, there are other conditions that induce GSH synthesis in other cells, and they may be of relevance to lung cells.

A potential role for GSH has been shown in the modulation of c-*fos* and c-*jun* gene expression by cigarette smoke condensate in Swiss 3T3 fibroblasts and conducting airway epithelium (144, 172). The c-*fos* and c-*jun* genes belong to a family of stress- and differentiation-related immediate-early response genes, the expression of which generally represents the first measurable response to a variety of chemical and physical stimuli (174). Cigarette smoke condensate exposure led to the induction of the c-*fos* gene, and this effect was

Table 1. *Inducers of glutathione and γ-glutamylcysteine synthetase*

Agent	Cell Line/Organ	Reference No.
Oxidants		
Hydrogen peroxide	A549 alveolar type II cells	198, 203
Menadione	A549 cells, rat lung L2 cells	136, 198
Cigarette smoke	A549 cells, rat lungs	129, 199
Dimethylnaphthoquinone	Rat lung L2 cells	80, 136, 242
Xanthine/xanthine oxidase	A549 alveolar type II cells	197
t-Butylhydroquinone	HepG2 cells	81, 82
4-Hydroxy-2-nonenal	Rat L2 cells	135
Ozone	Rat and monkey lungs	66
Hyperoxia	A549 cells, rat lungs	197, 257
Phenolic antioxidants		
β-Napthoflavonone	HepG2 cells	161, 170
Apocynin	A549 alveolar type II cells	125
Butylated hydroxyanisole	Mouse liver and kidney	69
Butylated hydroxytoluene	Mouse liver and kidney	248
Pyrrolidine dithiocarbamate	HepG2 cells	262
5,10-dihydroindeno(1,2-*b*)indole	Mouse hepatoma cells	137
Cytokines		
TNF-α	HepG2 cells, A549 cells	164, 196
IL-1β	Mouse vascular endothelial cells	250
Nitric oxide donors		
S-nitroso-*N*-penicillamine	Rat aortic muscle cells	158
DETA NONOate	Rat lung fibroblasts	261
Nitric oxide	Bovine aortic endothelial cells	159
Radiation		
Ionizing radiations	Glioblastoma cells, HepG2 cells	103, 165
Metals		
Selenium	Rat liver	46
Iron	Mouse liver	183
Cadmium	A549 alveolar type II cells	96
Mercury	Rat kidney	128, 265
Arsenite	Mouse peritoneal macrophages	12
Chemotherapeutic agents		
Cisplatin	Human ovarian cell line	88, 224, 266
Melphalan	Human multiple myeloma cells	168
Others		
Heat shock	K562 erythroid cells	118
Oxidized low-density lipoprotein	Human vascular endothelial cells	45

TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β.

mimicked by peroxynitrite and smoke-related aldehydes in concentrations that are present in cigarette smoke condensate (174, 175). The effects of cigarette smoke condensate can be enhanced by pretreatment of the cells with DL-buthionine-(*SR*)-sulfoximine (BSO) to decrease intracellular GSH and can be prevented by treatment with NAC (173). Thus depletion of GSH by cigarette smoke condensate leads to induction of c-*fos* and c-*jun*, components of AP-1, which may then act to induce γ-*GCS-HS* gene expression as a feedback mechanism.

Oxidative stress produced by hyperoxia, ozone, xanthine/xanthine oxidase, H_2O_2, menadione, lipid peroxidation products (4-hydroxy-2-nonenal), oxidized low-density lipoprotein, ionizing radiation, BSO, and heat shock leads to sustained increases in GSH levels by upregulation of γ-*GCS-HS* mRNA in alveolar epithelial cells in vitro and in vivo in lungs (45, 118, 136, 165, 242, 257). NO and its donors such as *S*-nitroso-*N*-penicillamine or DETA NONOate cause a transient depletion of GSH followed by induction of GSH synthesis by enhanced expression of γ-*GCS-HS* in rat aortic vascular smooth muscle cells (158), pulmonary fibroblasts (261), and bovine aortic endothelial cells (159). The increase in GSH caused by NO donors is a further potential mechanism to protect cells against oxidative stress. γ-*GCS-LS* is also concomitantly induced in response to oxidants and phenolic antioxidants in rat lung epithelial L2 cells and liver HepG2 cells, suggesting that concomitant induction of both subunits may provide a potential mechanism to enhance cellular GSH synthesis and so develop cellular tolerance to oxidative stress (81, 168, 242). Support for this comes from studies (136, 171) of rat epithelial L2 cells exposed to sublethal oxidative stress that showed increased GSH content associated with the development of tolerance to further oxidant assault in these cells. Furthermore, ozone exposure in rats and monkeys was associated with an initial decrease in GSH followed by a significant increase in GSH levels in airway epithelial cells (66). The increase in GSH levels was associated with tolerance of the airway cells to further oxidative stress (66).

In rabbits, exposure to hypoxia-reoxygenation decreases lung GSH content associated with an increase in GSSG levels (104). Oxidative stress imposed by heavy metals such as selenium (46), iron (183), methylmercury (265), sodium arsenite (12), and cadmium (12, 96) also induces GSH synthesis in various organs in both rats and mice. These metals may activate AP-1, induce protein phosphorylation (236), and activate c-Jun NH_2-terminal kinase (249). All these phenomena may be linked to the induction of γ-*GCS* expression. Other cytotoxic agents such as radiation (165) and chemotherapeutic agents such as cisplatin (88, 224, 266) and melphalan (168) that act through the generation of reactive oxygen species (ROS) also increase GSH synthesis in cancer cell lines. However, it is possible that GSH synthesis and a tolerance mechanism in response to various stimuli described in various cell lines may differ in lung epithelial cells.

ROLE OF PHENOLIC ANTIOXIDANTS IN THE REGULATION OF GSH SYNTHESIS

Gene regulation by phenolic antioxidants has been demonstrated to be the result of enhanced transcription factor binding to a *cis*-acting element known as the ARE or electrophile response element. The sequences for *cis*-acting ARE regions contain AP-1 or AP-1-like

elements in the consensus region (106). It has been demonstrated that the AP-1 or ARE sites are critical in the regulation of γ-*GCS* subunit genes (82, 161, 170, 196, 203, 224, 243). Exposure to phenolic antioxidants such as dietary 3-*t*-butyl-4-hydroxyanisole and butylated hydroxytoluene as well as the synthetic indolic antioxidant 5,10-dihydroindeno(1,2-*b*)indole leads to induction of γ-*GCS* in mouse liver and kidney cell lines (69, 137, 248). The plant-derived phenolic antioxidant apocynin (4-hydroxy-3-methoxyacetophenone) also induces GSH synthesis in human alveolar epithelial cells (125). These effects of phenolic antioxidants are associated with the activation of mitogen-activated protein kinases, AP-1, and ARE (106, 179). Therefore, in addition to their scavenging abilities, phenolic antioxidants may provide additional protection from oxidant-induced injury by upregulating the expression of γ-*GCS* and increasing GSH. More recently, pyrrolidine dithiocarbamate, a sulfhydryl-modifying antioxidant compound possessing both antioxidant and prooxidant properties, has been shown to enhance DNA binding and transactivation of AP-1 and induce γ-*GCS-HS* and γ-*GCS-LS* gene expression, resulting in de novo GSH synthesis in liver HepG2 cells (262). Hence many direct or indirect oxidant stresses lead to an increase in GSH synthesis and, consequently, tolerance of further oxidative stress. Further identification and characterization of the types of naturally occurring and synthetic phenolic antioxidant compounds, which could act as potent inducers of the γ-*GCS* subunits, should aid in the development of effective pharmacological strategies for antioxidant treatment involving GSH regulation in airway disease.

ROLE OF DIETARY AMINO ACIDS IN THE REGULATION OF LUNG GSH

Dietary GSH and cysteine are absorbed intact in the small intestine and increase GSH levels in plasma and various tissues (94, 95, 239). Oral administration of GSH (100 mg/kg) in mice detected higher levels of GSH in the plasma within 30 min of administration (9). This was associated with substantially increased GSH concentrations in various organs including the lungs. The regulation of tissue GSH concentration by diet and nutritional status and the potential to restore GSH in humans have been reviewed in detail (23).

Deneke et al. (61) and other investigators (67) have reported that total lung GSH is dependent on the amount of sulfur-containing amino acids, particularly the level of cysteine in the diet. This observation is supported by nutritional experiments showing that the availability of cysteine is a limiting factor for GSH synthesis in cases where the diet is deficient in sulfur-containing amino acids (60). GSH levels in the lungs from rats on a protein-deficient diet supplemented with cysteine were lower than those in control rats but increased more rapidly than those in control rats after exposure to hyperoxia (59). Similarly, GSH supplementation to preterm rabbits attenuated the changes in lung mechanics and injury caused by hyperoxia (30). The requirement of dietary cysteine in GSH synthesis was confirmed in rats fed protein-deficient diets, which produced enhanced toxicity, with a failure of elevation in lung GSH levels on exposure to hyperoxia. This observation may have implications in smokers where less dietary intake of sulfur-containing amino acids is associated with abnormal cellular function and possibly low lung function (31). Replenishment of sulfur-containing amino acids in the protein-deficient diets elevated lung GSH and prevented enhanced oxygen-mediated toxicity or inflammation (77, 101). In vivo studies of GSH levels in the lung and other organs are complicated by the fact that there is considerable diurnal fluctuation of GSH levels in the various organs and that the fluctuations are not synchronized (14). For example, lung GSH levels fluctuate by 200% in rats. Thus, in addition to cystine transport, the nutritional requirement of cysteine, particularly in smokers, is an important step in the regulation of GSH in lungs in vivo.

Dietary regulation of the key enzymes involved in the synthesis of GSH has been demonstrated in the rat liver (15). Rats fed a basal low-protein diet for 2 wk had lower activity of γ-GCS. This suggests that diet plays an important role in the regulation of GSH biosynthesis. The lower enzyme activity was associated with lower expression of γ-*GCS-HS* and γ-*GCS-LS* in the rat liver, implicating the potential role of diet (protein or sulfur-containing amino acids) in the regulation of γ-*GCS* expression.

The biological levels of GSH may also depend on the quality of the food and its processing and preservation. Jones et al. (107) measured the concentration of total GSH in various food samples. They found that dairy products, cereals, and breads are generally low in GSH; fruits and vegetables have moderate to high amounts of GSH; and freshly prepared meats are relatively high in GSH. Generally, frozen foods are thought to contain similar levels of GSH as fresh foods, whereas other forms of processing and preservation may result in an extensive loss of GSH (107).

MITOCHONDRIAL GSH AND OXIDATIVE STRESS

Ten to twenty percent of the intracellular GSH is in the mitochondria and a small percentage is in the endoplasmic reticulum (78). The mitochondrial GSH pool is solely derived from the activity of a mitochondrial transporter that translocates GSH from the cytosol to the mitochondrial matrix (156). Mitochondria do not possess the enzymes γ-GCS or γ-GT (156). Mitochondria normally produce a substantial quantity of ROS (e.g., H_2O_2 and $O_2^-\cdot$), which are normally broken down by GSH-dependent peroxidase-catalyzed reactions. Hence it is possible that the generation of ROS either endogenously or under oxidative stress may partly be regulated by mitochondrial GSH. Mitochondrial GSH deficiency leads to injury to lung cells and lamellar body formation (150). Animals treated with BSO, an inhibitor of γ-GCS, show a low cytosolic GSH level and a 40% decrease in mitochondrial GSH levels in the cells

(150). However, Smith and Anderson (231) have reported that there is no relationship between mitochondrial GSH levels and the susceptibility to oxygen-induced lung damage in mice. This study, however, was performed in whole lung tissue, and it may be that individual cells such as alveolar epithelial and capillary endothelial cells are susceptible to oxidant-induced damage.

Mitochondrial GSH may also be susceptible to the oxidative stress imposed by TNF-α and by products of chemotherapeutic drug metabolism in various cell lines and in human lungs (213, 222). TNF-α is known to deplete cytosolic GSH levels transiently in lung epithelial cells (196). This depletion by TNF-α is thought to be due to oxidative stress from mitochondrial generation of $O_2^-\cdot$ in the electron transport chain (190). Cigarette smoke, which contains many electrophilic compounds and ROS, also depletes cytosolic GSH levels in alveolar epithelial cells in vitro and in lungs in vivo (129, 200) and mitochondrial DNA mutation in human lungs (76). It is likely that mitochondrial GSH plays a key role in maintaining cellular antioxidant defense system and thus cell integrity under conditions of various oxidative stress. Recent studies (6, 182) have shown that mitochondrial gene transfer of glutathione reductase and overexpression of *GPx* in various cell lines provided protection against oxidative stress. This suggests that the GSH redox system and its enzymes such as glutathione reductase and GPx may be important in the protection of mitochondrial and cellular functions under oxidative stress such as cigarette smoke in the lungs.

OXIDANT-ANTIOXIDANT IMBALANCE IN SMOKERS AND PATIENTS WITH COPD: ROLE OF LUNG GSH

Cigarette smoking is the main etiological factor in COPD (Fig. 3) (201, 202). The cellular mechanisms by which cigarette smoke-mediated free radicals are dealt with in the lungs and the adaptive response systems are likely to vary between individuals. Cigarette smoke is a complex mixture of over 4,700 chemical compounds, including high concentrations of free radicals and oxidants (47). Short-lived oxidants such as $O_2^-\cdot$ and NO are predominantly found in the gas phase. NO and $O_2^-\cdot$ immediately react to form the highly reactive peroxynitrite ($ONOO^-$) molecule. The radicals in the tar phase of cigarette smoke are organic in nature, such as the long-lived semiquinone radicals that can react with $O_2^-\cdot$ to form hydroxyl radical ($\cdot OH$) and H_2O_2 (194). The aqueous phase of cigarette smoke condensate may undergo redox recycling for a considerable period of time in the ELF of smokers (178, 201, 202, 268). The oxidant burden in the lungs is further enhanced in smokers by the release of ROS from macrophages and neutrophils (98, 143, 221). Increased sequestration of neutrophils in the pulmonary microcirculation occurs during acute smoking (147) and in acute exacerbations of COPD (225). In both of these circumstances, neutrophils have been shown to have decreased deformability (65, 225), probably due to an oxidant-mediated effect on neutrophil actin, enhancing neutrophil sequestration in the pulmonary circulatory bed and augmenting oxidant injury. Circulating neutrophils from cigarette smokers and patients with exacerbations of COPD release more $O_2^-\cdot$ (202, 205).

Smoking and exacerbations of COPD result in a decreased antioxidant capacity in plasma (201, 205, 206) in association with depleted protein sulfhydryls in the plasma (205, 206). Using an in vitro model, Eiserich et al. (70) showed that exposure of gas-phase cigarette smoke caused considerable depletion of antioxidants including ascorbate, urate, ubiquinol-10, α-tocopherol, and a variety of carotenoids, including β-carotene, which was associated with lipid peroxidation and protein carbonyl formation in plasma. They suggested that the α,β-unsaturated aldehydes (acrolein and crotonaldehyde) abundantly present in cigarette smoke may react with protein-SH and -NH_2 groups, leading to the formation of a protein-bound aldehyde functional group, and are capable of converting tyrosine to 3-nitrotyrosine and dityrosine. Exposure of human erythrocytes to filtered cigarette smoke in vitro also depleted intracellular GSH and protein thiols without any change in the concentration of ascorbate (148). Further support for the presence of systemic oxidative stress comes from the increased levels of H_2O_2 and of lipid peroxidation products such as isoprostane $F_{2\alpha}$-III in plasma, breath condensate, and urine in patients with exacerbations of COPD (55, 162, 193, 201, 202, 205). NO and peroxynitrite-mediated formation of 3-nitrotyrosine in plasma and free catalytic iron (Fe^{2+}) levels in ELF are elevated in

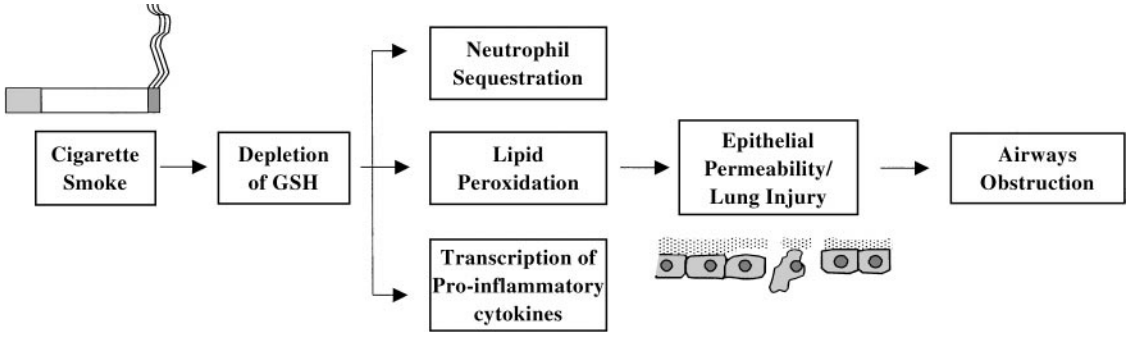

Fig. 3. Mechanisms of cigarette smoke-mediated lung injury. Lung damage is mediated by oxidants and free radicals present in cigarette smoke and is released by activated neutrophils. Cigarette smoke-mediated depletion of lung GSH leads to increased lipid peroxidation and transcription of proinflammatory cytokine genes that are involved in pathogenesis of chronic obstructive pulmonary disease.

Table 2. *Indexes of increased oxidative stress in COPD*

Biochemical Marker	Reference No.
Elevated breath hydrogen peroxide and 8-isoprostane levels	55, 162
Decreased plasma antioxidant capacity	205, 206
Elevated plasma lipid peroxides (TBARS) levels	205, 206
Elevated levels of plasma protein carbonyls	205
Plasma protein sulfhydryl oxidation	205, 205
Increased exhaled carbon monoxide	53
Release of ROS from peripheral blood neutrophils and alveolar macrophages	133, 206
Increased bronchoalveolar lavage fluid xanthine/xanthine oxidase activity	192
Increased urinary isoprostane $F_{2\alpha}$-III levels	193

COPD, chronic obstructive pulmonary disease; TBARS, thiobarbituric acid-reactive substances; ROS, reactive oxygen species.

chronic smokers (124, 187). These direct and indirect studies indicate that an increased systemic and local pulmonary oxidant burden occurs in smokers and in patients with COPD (145). Many of these markers of oxidative stress have the potential to oxidize thiol groups such as GSH and contribute to the changes in GSH in smokers and patients with COPD (Table 2).

MOLECULAR REGULATION OF LUNG GSH IN SMOKERS AND PATIENTS WITH COPD

There are few measurements of GSH in the lung ELF of smokers and patients with COPD (Table 3). Linden and colleagues (132, 133) showed that the severity of airway obstruction, as measured by FEV_1 in smokers with COPD, correlated negatively with the concentration of GSH in bronchoalveolar lavage (BAL) fluid (BALF): the higher the BALF GSH, the lower the FEV_1. The GSH concentration in BALF from patients with COPD was similar to that in chronic smokers with no airflow obstruction (133). This emphasizes the effects of smoking on GSH metabolism rather than reflecting the disease severity in the COPD patients. The actual relevance of these studies in the lungs of these patients is not known. It is possible that BALF GSH levels are influenced by the recent smoking of these patients (166). Behr et al. (16) recently showed that decreased GSH levels in BALF cells of chronic smokers was associated with a decreased expression of γ-GCS-LS without a change in γ-GCS-HS expression. This highlighted the fact that increased GSH levels in the ELF of chronic smokers was not associated with increased GSH levels in alveolar macrophages. These studies suggested that the increased GSH levels that are found in the ELF of chronic smokers may provide a protective adaptive mechanism, whereas the most injurious effects of cigarette smoke may occur repeatedly during and immediately after acute cigarette smoking when the lung is depleted in GSH.

Table 3. *GSH concentration in lung lining fluids of smokers and patients with COPD*

	Nonsmokers	Chronic Smokers	COPD	Reference No.
BALF, μM	2.26 ± 0.73	6.64 ± 1.98		166
ELF, μM	353.7 ± 125.3	762.7 ± 257.1		166
ELF, μM	339 ± 112	544 ± 97.6		42
BALF, μM	0.16 ± 0.02	0.3 ± 0.02	0.39 ± 0.04	132, 133

Values are means ± SE. GSH, glutathione; BALF, bronchoalveolar fluid; ELF, epithelial lining fluid.

The ability of individuals to regulate antioxidant defense mechanisms, such as the level of GSH in response to cigarette smoke, may be genetically determined. Variations in these protective responses may, in part, relate to why only 15–20% of smokers develop COPD (232). Further studies are needed to unravel the molecular mechanisms that regulate GSH in smokers and patients with COPD to assess the role of GSH as a protective mechanism in these conditions. A study (128) in the rat kidney with in situ hybridization revealed that γ-*GCS*, which is induced in response to methylmercury, is mainly localized in renal cortical cells. Similar techniques may be useful in studying GSH regulation in animal models and in resected lung specimens in smokers and patients with COPD.

EFFECT OF CIGARETTE SMOKE AND ITS CONDENSATE ON GSH REDOX SYSTEM IN LUNGS

There are limitations in extrapolating results obtained by the use of different experimental conditions and/or protocols to study the biological effects of cigarette smoke because no standard protocol is used. The results obtained from many varied studies may differ due to the experimental conditions such as the use of different brands of cigarettes, the preparation of cigarette smoke condensates or extracts, storage conditions, and exposure dose to the cells. In the present review, we distinguished the studies of whole cigarette smoke and its gas phase and condensates or extracts used by various investigators.

GSH and its redox system are important for the detoxification of toxic metabolites and lipid peroxides in lung tissue. Acute exposure of whole cigarette smoke or its condensate to alveolar epithelial cells in culture or to lungs in vivo in rats and rabbits has been shown to deplete intracellular GSH, which is associated with the formation of GSH conjugates, without any significant oxidation of GSH to GSSG (109, 129, 160, 200). However, chronic inhalation of cigarette smoke in rats was associated with a dramatic depletion of GSH, with a significant increase in the levels of GSSG and protein S-thiolation in the lung (185, 233). Cigarette smoking may also affect critical detoxifying and regulatory enzymes such as GPx, glutathione reductase, and GST involved in the GSH redox system in lungs. The acute exposure of alveolar epithelial cells in vitro and of the lungs of rats and rabbits to cigarette smoke or its condensate resulted in a reduction in the activities of GPx and glucose-6-phosphate dehydrogenase, without any significant change in the activity of glutathione reductase and GST (109, 160, 200, 216). In an another study, when young rats were chronically exposed to cigarette smoke for 21–30 days, the activities of GPx, glutathione reductase, and glucose-6-phosphate dehydrogenase were increased in the lungs compared with

those in nonexposed rats (267). The increase in enzymatic activity may be associated with a tolerance to cigarette smoking. However, alveolar macrophages or lung tissue obtained from chronic smokers did not show any appreciable changes in the activity of GPx or GST (154, 186).

IMPORTANCE OF GSH IN PROTECTING AGAINST EPITHELIAL PERMEABILITY AND LUNG INJURY

The epithelium lining the airways and alveoli has a protective barrier function. In response to injury, the epithelium loses its selective permeability and becomes more permeable to the movement of water, ions, and macromolecules. The lower respiratory tract is particularly sensitive to injury from inhaled and locally produced oxidants.

Airway Epithelium

Alveolar cells are normally covered with a thin protective layer of epithelial fluid, which is rich in antioxidants such as GSH, mucin, urate, and ascorbate (52). In addition, extracellular GPx, which has recently been described (8), is secreted into the ELF by alveolar epithelial cells and macrophages and may provide a further defense against oxidants. After acute cigarette smoke-oxidative stress, the ELF may become depleted of antioxidants such as GSH and extracellular GPx, increasing the potential for damage to the underlying epithelial cells. Recent data (166) indicated that GSH is increased twofold in the ELF of chronic smokers compared with that in nonsmokers, but this does not occur after acute smoking (Fig. 4). However, despite the increased levels of GSH in ELF, it appears to be insufficient to protect against cigarette smoke-induced epithelial cell damage.

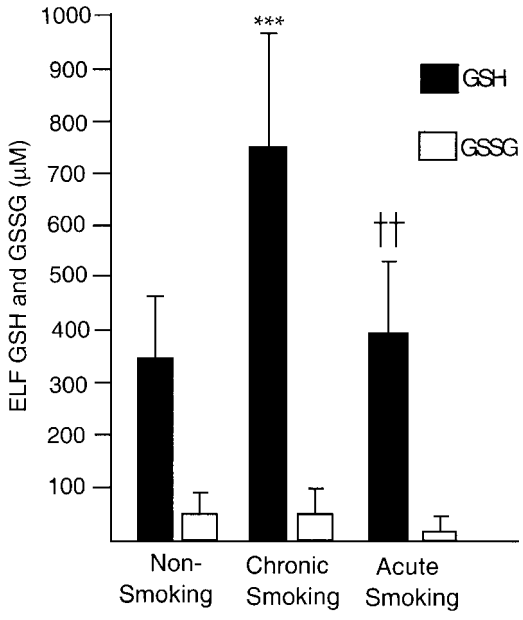

Fig. 4. GSH and GSSG in epithelial lining fluid (ELF) in nonsmokers and chronic and acute smoking groups. Values are means ± SE. *** $P < 0.001$ compared with nonsmokers. †† $P < 0.01$ compared with chronic smokers.

In vitro experiments with cultured epithelial cells showed a dramatic depletion of intracellular levels of GSH with exposure to cigarette smoke condensate, and this GSH depletion may cause increased air space epithelial permeability (123, 131). Furthermore, decreasing GSH levels in these cells with the γ-GCS inhibitor BSO also produced increased epithelial permeability (131). The presence of extracellular GSH in concentrations of 500 μM, which are similar to concentrations present in lung ELF, totally protected against the increased epithelial permeability that is produced by cigarette smoke condensate in in vitro experiments (129). Similarly, it has been reported that incubation with extracellular GSH (500 μM) and increasing intracellular GSH protected against other oxidant-mediated injury in alveolar type II cells and lung fibroblasts (28, 29, 93, 97, 241).

The role of intracellular GSH in cigarette smoke-induced epithelial permeability and inflammation is also evidenced in human bronchial epithelial cells and epithelial and endothelial cells (153, 176, 220). Direct exposure of human bronchial epithelial cells and endothelial cultures to cigarette smoke led to a markedly significant dose- and time-dependent increase in the permeability and detachment of these cells, and this effect was abrogated when the cells were treated with GSH or NAC (176, 220). Recent data (5, 99, 153) have shown that exposure to cigarette smoke extracts produced apoptosis in a dose- and time-dependent manner and the release of neutrophil and monocyte chemotactic activities in A549 epithelial cells and rat alveolar macrophages. This cytotoxic effect was associated with a decrease in the level of intracellular GSH, and NAC treatment protected against the toxic effects of cigarette smoke. Other toxic compounds such as asbestos enhanced cigarette smoke-mediated GSH depletion and epithelial injury in cultured rat airway epithelial cells (110). These studies suggested that GSH has a critical role in maintaining epithelial membrane integrity and an important role in protection against cigarette smoke- or oxidant-mediated epithelial injury.

Within Lungs

Rodents exposed to cigarette smoke or to cigarette smoke condensate instilled intratracheally showed a profound decrease in GSH in both ELF and the lung (129, 200, 216). This decrease in lung GSH appears to be associated with an increase in air space epithelial permeability (123, 131). Decreasing GSH levels in vivo in the rat with the γ-GCS inhibitor BSO produces increased epithelial permeability (131). Nishikawa et al. (181) recently demonstrated that acute cigarette smoke exposure in guinea pigs produced lung inflammation due neutrophil influx into the airways, which was associated with NF-κB activation and a higher expression of interleukin-8 mRNA in alveolar macrophages and, to a lesser extent, in alveolar epithelial cells. This effect may be due to depletion of lung GSH and increased lipid peroxidation by cigarette smoke. Furthermore, Li et al. (130) have reported that instillation of air particulate matter < 10 μm into the lungs of rats

caused inflammation, decreases in lung GSH levels, and increases in epithelial permeability. Cigarette smoke may potentiate this effect on lung GSH depletion. These studies in animal models are supported by a study (166) in vivo in humans that indicated that increased epithelial permeability occurs acutely after cigarette smoking. This increased epithelial permeability, which occurs in smokers, may enhance the access of proteases to the lung interstitium.

ROLE OF GSH IN PROTECTION AGAINST OXIDANT INACTIVATION BY ANTIPROTEASES

GSH may act to protect lung tissue from excessive proteolytic activity. There is evidence that the antiproteinase protective shield in the lungs may be weakened by ROS. Leukocytes contain a number of potent proteinases that are released into the extracellular environment. Most of the major human antiproteinases such as α_1-proteinase inhibitor (α_1-PI) and secretory leukoproteinase inhibitor may be inactivated by activated neutrophils by an oxidant-dependent mechanism (43, 120). A significant fall in elastase inhibitory capacity in BALF was reported after acute smoking (2). However, inconclusive results that may be due to the improper control of smoking history were obtained in chronic smokers. In vitro studies demonstrated that cigarette smoke gas and aqueous phases can both decrease the elastase inhibitory capacity of α_1-PI (48, 73, 74). Studies have demonstrated that α_1-PI recovered from the lungs of cigarette smokers and cigarette smoke-exposed animals is partially inactivated and that methionine residues of α_1-PI recovered from the ELF from lungs of cigarette smokers are oxidized (44). However, another study (167) has failed to show these differences, and the matter remains controversial (167). A study (84) in which aerosolized secretory leukoproteinase inhibitor was administered to sheep showed increased postaerosol levels of GSH in the ELF, suggesting another role for GSH in the redox maintenance of alveolar fluid.

MODULATION OF GSH BY PROINFLAMMATORY MEDIATORS TNF-α, TRANSFORMING GROWTH FACTOR-β1, AND ANTI-INFLAMMATORY AGENTS AND GLUCOCORTICOIDS IN LUNG CELLS: IMPLICATIONS IN COPD

Proinflammatory Mediators

TNF-α is a ubiquitous proinflammatory cytokine and is recognized as an important mediator of inflammatory events in the lungs. It induces chronic inflammatory changes associated with an increase in a variety of defense mechanisms including antioxidants (264). TNF-α induces oxidative stress by the generation of ROS via leakage from the mitochondrial electron transport chain and therefore depletes GSH in human alveolar epithelial and pulmonary artery endothelial cells (190, 196). The mechanism of GSH depletion by TNF-α has been proposed to be upstream from the ceramide and sphingomyelinase pathways, suggesting that a signaling mechanism is involved in this event (134). TNF-α is an important inflammatory mediator in COPD and is present in elevated levels in the BALF and sputum from these patients (114). TNF-α initially decreased GSH levels, followed by a rebound increase in human alveolar epithelial cells and liver HepG2 cells (164, 196). This induction of GSH synthesis by TNF-α is mediated by AP-1 (196). TNF-α and interleukin-1β also upregulate γ-*GCS-HS* mRNA in mouse vascular endothelial cells (250). These events have relevance in vivo because patients with acute respiratory distress syndrome, who exhibit increased plasma oxidative stress, also have elevated TNF-α concentrations, and this is associated with a higher plasma GSH concentration (121). Furthermore, a strong correlation between systemic TNF-α levels and increased GSH levels was found in these patients. In an animal model, a chronic intraperitoneal injection of TNF-α or endotoxin in low protein-fed rats resulted in a rebound recovery of lung GSH and the activities of glutathione reductase and GPx, suggesting a role for the GSH redox system in metabolism during inflammation (91, 101). A recent study by Jaeschke et al. (105) demonstrated that acute endotoxin treatment in mice led to increased plasma levels of GSSG, and this was further pronounced in GPx-deficient mice when TNF-α was injected and subsequently resulted in hepatic parenchymal cell injury. However, the regulation of GSH levels in human lungs during chronic inflammation is not known.

Anti-inflammatory Agents and Hormones

Glucocorticoids such as dexamethasone are widely used as anti-inflammatory agents in various lung diseases including exacerbations of COPD. Airway epithelium is one of the most important targets for inhaled glucocorticoids in lung diseases. Dexamethasone when added to A549 cells decreases both basal and stimulated (TNF-α-treated) GSH levels in these cells (196, 197). Dexamethasone also decreases γ-*GCS-HS* gene expression in alveolar epithelial cells in vitro by a transcriptional mechanism involving the inhibition of AP-1 transcription factor (196). Therefore, the use of dexamethasone in patients with inflammatory lung diseases may prevent synthesis of the protective antioxidant GSH, which may be attributed to an interaction between the glucocorticoid receptor and AP-1 or an oxidative inactivation of glucocorticoid-receptor binding in lung cells. This may have implications for the lack of effectiveness of long-term treatment with corticosteroids in COPD.

Various hormones can regulate γ-GCS activity and γ-GCS-HS expression (37, 139). Insulin and hydrocortisone treatment produced a significant increase in the activity of γ-GCS and γ-*GCS-HS* but not of the γ-*GCS-LS* mRNA level in cultured hepatocytes (36, 139). This observation is confirmed in vivo where the liver exhibited lower GSH levels and γ-GCS activity in insulin-deficient diabetic or adrenalectomized rats (37, 139). These studies suggested that hormones are required in GSH synthesis in the liver. In contrast, GSH synthesis and γ-GCS activity were acutely inhibited by hormone-mediated activation of various signal transduction path-

ways in vivo in the rat liver (140). Thus in vitro and in vivo findings in cultured hepatocytes and the intact liver may be specific and may not operate exactly in a similar fashion in other tissues or cell types because glucocorticoid administration in rats did not affect hepatic GSH levels or γ-GCS activity (139).

Growth Factors

Transforming growth factor (TGF)-β1 is a multifunctional growth factor that modulates cellular proliferation and induces differentiation and synthesis of extracellular matrix proteins, including collagens and fibronectin in many types of lung cells (21). A recent study (54) has shown increased expression of TGF-β1 in bronchiolar and alveolar epithelia in COPD patients compared with that in healthy subjects. TGF-β1 also downregulates γ-*GCS-HS* mRNA and GSH synthesis in human alveolar epithelial cells and pulmonary artery endothelial cells in vitro (7, 260). Interestingly, a recent study by Factor et al. (75) showed decreased GSH synthesis in a TGF-transgenic (overexpression) mouse model. These transgenic mice showed susceptibility to oxidant-mediated injury (75). It has been recently shown that γ-*GCS-HS* mRNA expression is under the control of the AP-1 transcription factor (196, 203). TGF-β1 may decrease γ-*GCS-HS* gene expression via an AP-1 mechanism (251). Thus higher levels of TGF-β1 may downregulate GSH synthesis in the lungs of patients with COPD. In addition, decreased GSH levels may also have direct functional consequences. An in vitro study (41) showed that GSH (in the concentration range normally found in ELF) suppressed fibroblast proliferation.

GENETIC REGULATION OF GSH SYNTHESIS IN SMOKERS AND PATIENTS WITH COPD

Genetic susceptibility to cigarette smoke has been suggested as a risk factor for the development of COPD (119). Recently, it has been shown that polymorphic expression of several different xenogenes, including *CYP1A1*, GSTs (*GST-M1* and *GST-P1*), and microsomal epoxide hydrolase (*mEPHX*), are associated with an increased risk of chronic lung diseases (38, 229). Cigarette smoke is a complex mixture of chemical compounds, including high concentrations of free radicals and other oxidants that are capable of being transformed into GSH conjugates directly or by indirect metabolism by the GST enzyme system (208). Variations in the expression of the γ-*GCS* gene in humans may represent a new susceptibility factor in the oxidant-induced injury, which is thought to occur as part of the pathogenesis in COPD. It has been proposed that a GAG trinucleotide repeat polymorphism occurs in the 5′-coding and noncoding regions of the γ-*GCS-HS* gene (256). Genetic analysis of 50 unrelated Caucasians identified three alleles as follows: *A1* (9 repeats, 35% frequency), *A2* (8 repeats, 11% frequency), and *A3* (7 repeats, 54% frequency). Although certain trinucleotide repeats have been associated with recombinatory events, the functional significance of this particular allelic polymorphism, if any, is unknown.

The γ-*GCS* subunit genes are located on separate chromosomes, and the expression of their mRNAs varies considerably in different tissues (227, 228, 256). Human γ-*GCS-HS* is located at chromosome 6 (6p12) and γ-*GCS-LS* on chromosome 1 (1p21) (227, 228, 246). Genetic analysis reveals that a frequent deletion of γ-*GCS-LS* on chromosome 1p22 → p21 occurs in human malignant mesothelioma. This gene deletion is considered to predispose an individual to the development of mesothelioma (219). Recent data demonstrated that AP-1 and ARE/EpRE, which are present in the promoter region of γ-*GCS-HS* and γ-*GCS-LS* genes, may be directly involved in the regulation of GSH in human cells (81, 82, 161, 170, 203). Within a population, it is likely that there will be variation (gene deletion or mutation) in the 5′-coding or noncoding region of the γ-*GCS-HS* and γ-*GCS-LS* genes. Future studies need to be directed toward establishing the existence of such polymorphisms and whether any association exists between this polymorphism and the susceptibility to the development of COPD.

TREATMENTS TO AUGMENT LUNG GSH

It is now evident that oxidant-antioxidant balance, which plays an important role in the pathogenesis of COPD, is altered in favor of oxidants in smokers. GSH in the ELF and lung cells may counteract the immediate potential toxic and inflammatory effects of cigarette smoke free radicals. Therefore, it seems rational to pursue various strategies aimed at correcting lung and ELF GSH deficiency. The following GSH- or cysteine-delivering strategies are not only useful in the treatment of cigarette smoking-induced lung disease but also are beneficial in general in various inflammatory lung diseases where GSH levels have been shown to be altered (35, 40, 146, 217, 235).

There are two main ways of enhancing lung GSH: *1*) supplementation of GSH or its precursors and *2*) molecular manipulation of GSH biosynthesis by gene transfer.

Supplementation of GSH or Its Precursors

Supplementation of GSH. GSH itself is not efficiently transported into most animal cells. GSH in excess may be a source of thiol or thionyl radicals under oxidative conditions (215). Thus intracellular GSH concentrations are not easily manipulated by extracellular GSH. However, there are reports that exogenous GSH does increase intracellular GSH in vitro (63) and protects against oxidant stress in alveolar type II cells (28, 93). There are several theories on how this may occur. Extracellular GSH reacts with cystine to generate cysteine. Cysteine is transported efficiently across cell membranes and is used as a substrate for intracellular GSH synthesis in cultured lung cells (28, 63). Some epithelial cells can take up intact GSH by a Na^+-dependent uptake system that transports GSH into the cells against a concentration gradient (10, 29, 111), whereas other types of cells do not have this capacity (58, 252). Extracellular GSH may also be broken down

in the extracellular medium by γ-GT into the γ-glutamyl amino acid and the dipeptide cysteinylglycine. These two moieties can subsequently be taken up by the cells and used to resynthesize GSH by the actions of γ-GCS and glutathione synthetase (207).

Augmentation of the antioxidant screen in the lungs by GSH aerosol or nebulizer therapy has been tried in patients with IPF, mild asthma, and cystic fibrosis (22, 33, 149). GSH aerosol therapy normalized low GSH levels in the lungs of these patients (32). However, nebulized GSH had a detrimental effect in asthmatic patients by producing bronchoconstriction (149). GSH aerosol also increased the formation of GSSG in patients with IPF (22). Thus GSH aerosol therapy may not be an appropriate way of correcting GSH concentrations in the lung ELF.

Supplementation of GSH precursors. At present, GSH precursor amino acids are the best means of manipulating GSH biosynthesis intracellularly. Cysteine is a thiol-containing amino acid and is usually the rate-limiting amino acid in GSH synthesis (157). Cysteine administration is not possible because it is a neurotoxin and is also oxidized to cystine (113). Moreover, the addition of cysteine to a basal essential amino acid diet for mice led to weight loss and death (18). Many studies (11, 29, 111) have shown species and cell variations in cysteine and cystine transport. There are many GSH precursors, notably NAC, which is currently used to enhance GSH levels in the lung.

NAC. NAC, a cysteine-donating compound, acts as a cellular precursor of GSH and becomes deacetylated in the gut to cysteine after oral administration (184). NAC may also reduce cystine to cysteine, which is an important mechanism for intracellular GSH elevation in vivo in lungs. It reduces disulfide bonds but also has the potential to interact directly with oxidants. NAC is also used as a mucolytic agent (to reduce mucus viscosity and improve mucociliary clearance) (184). Pharmacological therapies, particularly with thiol antioxidants such as NAC, have been used in an attempt to enhance lung GSH in patients with COPD with varying success (24, 25, 202). NAC can be administered in oral, intravenous, and inhaled forms. When NAC is given orally in low dosages (600 mg/day) to patients with COPD and normal subjects, very low levels of NAC can be detected in the plasma for up to 2 h after administration (25). NAC does not accumulate in normal subjects on repeated administration (50). Bridgeman et al. (25) did show that after 5 days of three doses of NAC per day, there was a significant increase in plasma levels of GSH. However, there was no associated rise in the levels of GSH in the BALF or ELF nor was there a significant increase in lung tissue cysteine or GSH (25). These data seem to imply that producing a sustained increase in lung GSH (in ELF and lung tissue) is difficult with NAC and does not equate with an increase in plasma levels of GSH. This suggests that it may be difficult to produce a major change in GSH with NAC in subjects who are not already depleted in GSH. There have also been studies (20, 27) of patients with COPD where the administration of NAC has led to conflicting results on the number of exacerbations of COPD. This probably arose as a result of differing dosage regimens and disease severity in these studies. Eklund et al. (71) studied the effect of NAC in healthy chronic cigarette smokers after an 8-wk period of 200 mg three times daily. This produced a reduction in the BALF of eosinophilic cationic protein, lactoferrin, anti-chymotrypsin, and chemotactic activity for neutrophils. A multicenter study with NAC by metered-dose inhalers in patients with a chronic cough failed to show a positive effect on well being, sensation of dyspnea, cough, or lung function (68). Furthermore, a direct link between these clinical effects (i.e., reduction in the number of exacerbations and decline in lung function) and its antioxidative property as a mechanism of action has not been established so far.

There is a possibility that NAC may have a deleterious effect on alveolar macrophages (255). In vitro NAC treatment has been shown to inhibit neutrophil and monocyte chemotaxis and oxidative burst responses (115). Intravenous administration of NAC in humans has been associated with some potentially serious side effects. A low (but not a high) dose of NAC (150 mg/kg) in water two times daily in normal volunteers improved performance of human limb muscle during fatiguing exercise (211). However, a deleterious effect of NAC was also shown at higher doses (550 and 950 mg/kg for 2 days), leading to increased mortality in rats (234). The toxic effect of a high dose of NAC may be due to its prooxidant effects in the presence of transition metal ions such as Fe^{2+}, which might affect the redox status of various membrane proteins by reduction of protein disulfide bonds. Hence these studies suggest that the dose of NAC is crucial in designing antioxidant therapy to protect against cigarette smoke-mediated lung injury.

N-acystelyn. N-acystelyn (NAL), a lysine salt of NAC, is a mucolytic and antioxidant thiol compound. The advantage of NAL over NAC is that it has a neutral pH, whereas NAC is acidic. NAL can be aerosolized into the lung without causing significant side effects (177). Gillissen et al. (85) and Gillissen and Nowak (86) compared the effect of NAL and NAC and found that both drugs enhanced intracellular GSH in alveolar epithelial cells and inhibited hydrogen peroxide and $O_2^{-}\cdot$ released from human blood-derived polymorphonuclear neutrophils from smokers with COPD. NAL also inhibited ROS generation by human polymorphonuclear neutrophils induced by serum-opsonized zymosan. This inhibitory response was comparable to the effects of NAC (177). Therefore, NAL may represent an interesting alternative approach to augmenting the antioxidant screen in the lungs.

GSH esters. Most animal cells export GSH normally, but there is little, if any, evidence of the uptake of intact GSH (58, 63, 157). This has led to the search for derivatives of GSH that might be transported into cells and could thereafter be converted into GSH. GEE contains an ethyl group esterified to the glycine of GSH. This GSH ester is more lipophilic and enters cells more readily than GSH and is hydrolyzed to GSH by a cytosolic nonspecific esterase (117). Thus GEE provides

direct intracellular availability of GSH, bypassing the γ-GCS feedback inhibition by GSH (245).

GSH monoethyl ester (MEE) has been noted to be resistant to cleavage by the enzyme γ-GT (3, 4). MEE has been used to increase intracellular reduced GSH in vitro and has been shown in some studies to be more effective than GSH itself (3, 4, 245). The monoesters of GSH can protect cells in suspension against radiation (258) and animals against the effects of a variety of toxic compounds, including heavy metals (175), anticancer agents (240), and acetaminophen (195). MEE treatment given to BSO-treated adult mice led to an increase in cytosolic and mitochondrial GSH in the lungs and liver (151). A recent study (97) in rat lungs incubated with various GSH precursors revealed that GSH isopropyl diester and γ-glutamylcysteine isopropyl diester elevated GSH concentrations to a level above that of the feedback-inhibited level. However, GSH isopropyl monoester and γ-glutamylcysteine isopropyl monoester did not increase lung GSH levels (97). In vivo administration of γ-glutamylcysteinyl ethyl ester (160 μmol/kg) in mice provided protection of hepatocytes against carbon tetrachloride toxicity by maintaining high levels of hepatic cell GSH levels (180).

In vitro studies have shown that there is variability in the ability of different types of lung cells to synthesis GSH from its precursors (63, 93, 245). It is also important to note that the increase in intracellular thiols that is produced by exogenous reduced GSH, MEE, or NAC is short-lived and returns to the control level within 24 h. Therefore, in conditions where GSH is chronically depleted, it is difficult to maintain a sustained level of GSH in ELF or in lung cells.

Other precursors of GSH. Thiazolidines are potentially useful compounds for cysteine delivery. L-Thiazolidine-4-carboxylic acid is hydrolyzed to cysteine. L-2-Oxothiazolidine-4-carboxylate is another compound that is transported effectively into cells and is split enzymatically to give L-cysteine. This compound is more effective than NAC in protecting liver GSH in acetaminophen-treated mice (263). L-2-Oxothiazolidine-4-carboxylate is also able to protect cultured endothelial cells against hyperoxia-induced injury (244). GSH may also be spared by administration of ascorbate. A study (152) has shown that tissue GSH deficiency is restored by ascorbate supplementation. However, there is no study that has validated these compounds in clinical trials in humans.

Molecular Manipulation of GSH Biosynthesis by Gene Transfer in Lung Cells

The induction of γ-*GCS* by molecular means holds great promise in oxidant-mediated injury in lungs. Cellular GSH may be increased by increasing the activity of γ-GCS and glutathione synthetase by gene transfer techniques. With the use of a strain of *Escherichia coli*, overexpression of γ-*GCS* and *glutathione synthetase* provided more protection against radiation damage than the wild-type strain (163). Transfection of complementary DNAs of human γ-GCS-HS and γ-GCS-LS resulted in the elevation of intracellular GSH levels in COS-7 cells (169). These cells were thereafter resistant to chemotherapeutic drugs (169). Similarly, oxidative stress induces increased GSH levels and γ-GCS expression in lung cells, and these cells developed tolerance against further oxidative stress (171). The preparation of transgenic mice that have an increased capacity to catalyze GSH synthesis by increasing the activity of γ-GCS is now possible. Alternatively, the levels of both GSH synthetases may need to be increased. The possibility that transgenic animals of this type may, in addition, require increased dietary levels of cysteine or its precursors to maintain higher cellular levels of GSH also needs to be considered. Individuals who may have a genetic susceptibility would be expected to benefit from therapy that supplies the missing enzyme by gene transfer.

Knockout mice for the γ-*GCS-HS* and γ-*GCS-LS* genes will be useful in assessing the functional role or importance of GSH in protecting against cellular damage. Mouse cDNAs for γ-GCS-HS and γ-GCS-LS have been cloned and sequenced (112, 209, 210). Genetic mapping of both γ-*GCS-HS* and γ-*GCS-LS* has been described on mouse chromosomes 9D-E and 3H1–3, respectively (227). It is expected that these knockout mice will soon be made by targeted gene disruption. However, GSH knockout may be lethal (92). GSH depletion is also possible by using antisense nucleotides transfected to block γ-*GCS* subunit gene expression. Riboenzymes can be engineered to selectively cleave a specific RNA, thus modifying gene expression. Recently, a hammerhead ribozyme designed against the two γ-GCS subunits decreased intracellular GSH and inhibited γ-*GCS* subunit mRNA expression in mouse islet cells (116). It may be possible to use this ribozyme to confer γ-GCS-HS "knockout" at the mRNA level in cells in vitro. However, it is unclear whether this ribozyme is specific for lung cells. Bacterial model systems in *E. coli* lacking γ-*GCS-HS* and containing GSH mutants (*gshA*) are now available (126). These models may be useful in analyzing the GSH-associated cellular defense systems in response to oxidants in lung cells.

FUTURE CONSIDERATIONS OF THE THERAPEUTIC POTENTIAL OF GSH IN PATIENTS WITH COPD

Treatment of GSH deficiency by administration of GSH might be a logical and useful approach (155). It should also be noted that cellular protection is likely to depend not only on the cellular level of GSH at the time of challenge but also on the capacity of the cell to synthesize GSH and transport processes that control substrate availability. The normal intracellular concentration of GSH cannot be affected by the administration of exogenous GSH or amino acids and the peptide precursors for GSH because of feedback inhibition of γ-GCS by GSH. The administration of MEE may allow augmentation of intracellular GSH, but this may be toxic to cells at higher doses. Although many patients have been treated with NAC and GSH safely, no clinical trials have been conducted with the cell membrane-permeable derivative GSH esters. The dose necessary

to maintain elevated lung GSH levels in inflammatory lung diseases is also unknown.

Before any therapy begins with thiols, ex vivo and in vivo studies are required to provide information on GSH homeostasis in the lungs of smokers and patients with COPD. Future studies are required to study GSH regulation in the lungs of smokers with and without airway obstruction. This will increase our understanding of the transcriptional regulation of GSH synthesis in the lungs of nonsmokers, smokers, and patients with COPD, which will be useful for the development of molecular interventions in this condition. It is now reasonable to extend the duration and increase the dosage of therapy in pilot studies with compounds such as GEE, MEE, and NAL in patients with cigarette smoke-induced airway disease.

FUTURE DIRECTIONS AND CONCLUSIONS

Regulation of GSH synthesis is central to the control of cellular processes. Studies are needed to establish the relative importance of specific transcriptional control mechanisms under various conditions. It is also important to understand the possible coordinate expression and posttranslational regulation of the γ-GCS subunits with other enzymes and transport systems involved in GSH synthesis and metabolism. It is equally important to characterize the signal transduction pathways and specific *cis*-activating factors in the regulation of both *γ-GCS-HS* and *γ-GCS-LS* gene expression in response to specific stimuli.

It is clear that ROS contribute to the pathogenesis of COPD and that GSH is an important protective antioxidant in the lungs, which may be altered in this condition. Study of the role of GSH in protection against oxidative stress or inflammation in the lung cells of smokers and patients with COPD is an important area for further research. Modulation of intracellular thiol status by either molecular or genetic regulation of GSH synthesis in the lungs not only will buffer antioxidant potential but may also inhibit oxidant-mediated inflammatory responses.

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