

REVIEW

Oxidative stress and regulation of glutathione in lung inflammation

I. Rahman, W. MacNee

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ABSTRACT: Inflammatory lung diseases are characterized by chronic inflammation and oxidant/antioxidant imbalance, a major cause of cell damage. The development of an oxidant/antioxidant imbalance in lung inflammation may activate redox-sensitive transcription factors such as nuclear factor- κ B, and activator protein-1 (AP-1), which regulate the genes for pro-inflammatory mediators and protective antioxidant genes. Glutathione (GSH), a ubiquitous tripeptide thiol, is a vital intra- and extracellular protective antioxidant against oxidative/nitrosative stresses, which plays a key role in the control of pro-inflammatory processes in the lungs. Recent findings have suggested that GSH is important in immune modulation, remodelling of the extracellular matrix, apoptosis and mitochondrial respiration. The rate-limiting enzyme in GSH synthesis is γ -glutamylcysteine synthetase (γ -GCS). The human γ -GCS heavy and light subunits are regulated by AP-1 and antioxidant response elements and are modulated by oxidants, phenolic antioxidants, growth factors, and inflammatory and anti-inflammatory agents in lung cells.

Alterations in alveolar and lung GSH metabolism are widely recognized as a central feature of many inflammatory lung diseases such as idiopathic pulmonary fibrosis, acute respiratory distress syndrome, cystic fibrosis and asthma. The imbalance and/or genetic variation in antioxidant γ -GCS and pro-inflammatory *versus* antioxidant genes in response to oxidative stress and inflammation in some individuals may render them more susceptible to lung inflammation. Knowledge of the mechanisms of GSH regulation and balance between the release and expression of pro- and anti-inflammatory mediators could lead to the development of novel therapies based on the pharmacological manipulation of the production as well as gene transfer of this important antioxidant in lung inflammation and injury.

This review describes the redox control and involvement of nuclear factor- κ B and activator protein-1 in the regulation of cellular glutathione and γ -glutamylcysteine synthetase under conditions of oxidative stress and inflammation, the role of glutathione in oxidant-mediated susceptibility/tolerance, γ -glutamylcysteine synthetase genetic susceptibility and the potential therapeutic role of glutathione and its precursors in protecting against lung oxidant stress, inflammation and injury.

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Inflammation is an important protective response to cellular/tissue injury. The purpose of this process is to destroy and remove the injurious agent and injured tissues, thereby promoting tissue repair. When this crucial and normally beneficial response occurs in an uncontrolled manner, the result is excessive cellular/tissue damage that results in chronic inflammation and destruction of normal tissue. Reactive oxygen species (ROS), such as the superoxide anion liberated by phagocytes recruited to sites of inflammation, are proposed to be a major cause of the cell and tissue damage, including apoptosis, associated with many chronic inflammatory diseases [1–3]. Lung cells, in particular alveolar epithelial type II cells, are susceptible to the injurious effects of oxidants. It has been shown that lung cells release inflammatory mediators and cytokines/chemokines such as tumour necrosis factor- α (TNF- α), interleukin (IL)-1 and IL-8 in response to oxidative/nitro-

sative stress. The release of cytokines/chemokines induces neutrophil recruitment and the activation of key transcription factors such as nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1), thereby augmenting the inflammatory response and tissue damage [4, 5]. As a result, the acute and chronic alveolar and/or bronchial inflammatory response is a fundamental process involved in the pathogenesis of many lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), idiopathic pulmonary fibrosis (IPF) and cystic fibrosis (CF). The site and specific characteristics of the inflammatory responses may differ in each of these diseases, but all are characterized by the recruitment to the lungs and activation of inflammatory cells leading to an oxidant/antioxidant imbalance.

Glutathione (GSH) is a tripeptide (L- γ -glutamyl-L-cysteinyl-glycine) containing a thiol (sulphydryl) group. GSH

Respiratory Medicine Unit, ELEGI & Colt Laboratories, University of Edinburgh, Medical School, Edinburgh, UK.

Correspondence: I. Rahman
ELEGI Colt Research Laboratories
Wilkie Building
University of Edinburgh
Medical School
Teviot Place
Edinburgh EH8 9AG
UK
Fax: 44 1316511558

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is an important protective antioxidant against free radicals and other oxidants and has been implicated in immune modulation and inflammatory responses [6–8]. These events include modulation of redox-regulated signal transduction, regulation of cell proliferation, remodelling of the extracellular matrix (ECM), and antiprotease screen, apoptosis and mitochondrial respiration [7–12]. The antioxidant GSH has been shown to be critical to the lungs' antioxidant defences, particularly in protecting airspace epithelium (membrane integrity) from oxidative/free radical (cigarette smoke/air particulates)-mediated injury and inflammation [13–15]. Alterations in the levels of GSH in the lung lining fluid have been shown in various inflammatory conditions. For example GSH levels are decreased in the epithelial lining fluid (ELF) in IPF [16, 17], ARDS [18], CF [19], lung allograft [20] and human immunodeficiency virus (HIV)-positive patients [21]. In contrast, total glutathione concentrations, including the oxidized form (GSSG), are higher in the bronchial and alveolar fluid in patients with mild asthma (table 1) [22]. Glutathione is present in increased concentrations in the ELF of chronic smokers, whereas this is not the case in the ELF of acute smokers [23, 24]. However, GSH levels were not decreased in the ELF of IPF and HIV-positive patients who were smokers [25, 26]. A low GSH concentration in the ELF may contribute to an imbalance between oxidants and antioxidants in the lungs and may amplify inflammatory responses and potentiate lung damage.

It has been suggested that oxidants, antioxidants, and inflammatory and anti-inflammatory agents modulate the activation of redox-sensitive AP-1 and NF-κB [5]. AP-1 and AP-1-like antioxidant response element (ARE) have 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) region of both the human catalytic γ-GCS-HS and regulatory γ-GCS-LS genes contain putative AP-1 and ARE response elements which are necessary for γ-GCS expression in response to diverse stimuli [27–30]. It is possible that differences in ELF glutathione levels in various inflammatory lung diseases

are due to changes in the molecular regulation of GSH synthesis by AP-1 and ARE activation by oxidants and inflammatory and anti-inflammatory agents, its turnover/breakdown and/or transport in lung cells. However, the molecular mechanism of glutathione synthesis in lungs of patients with inflammatory lung diseases has not been studied. The imbalance and genetic variability of γ-GCS and pro-inflammatory gene expression in response to oxidative stress and inflammatory response may be a determinant of susceptibility to lung disease. The aims of this review are: 1) to describe the sources of oxidative stress in inflammation and the redox control of the transcription factors NF-κB and AP-1; 2) review the regulation, transport and metabolism of lung cell glutathione and γ-GCS gene expression in inflammation and oxidative stress; 3) discuss the possible role of γ-GCS *versus* pro-inflammatory gene imbalance in susceptibility/tolerance; and 4) assess the potential protective and therapeutic role of glutathione and other related thiols in oxidant-induced lung injury and inflammation.

Role of cell-derived oxidants in inflammation

The presence of oxidative stress in the airspaces and the blood initiates a number of early events during pulmonary inflammation. Inflammatory cells are sequestered into the pulmonary microcirculation and recruited to the airspaces as a result of the generation of mediators such as IL-8. Once recruited, inflammatory cells become activated and generate ROS in response to a sufficient level of a secretagogue stimulus (threshold concentration). The mechanism for this may involve neutrophil adhesion to endothelium and upregulation of CD18 integrins [31, 32], which is known to upregulate the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase hydrogen peroxide-generating system [1]. Activation of macrophages, neutrophils and eosinophils generates O₂^{•-}, which is rapidly converted to H₂O₂ by superoxide dismutase (SOD), and hydroxyl radicals, formed nonenzymatically in the presence of Fe²⁺ as a secondary reaction. In neutrophils, myeloperoxidase also catalyses the formation of the potent oxidant hypochlorous acid from H₂O₂ in the presence of chloride ions. ROS, which may also be released by lung epithelial cells [33, 34] may also stimulate inflammatory cells directly, thereby amplifying lung inflammatory and oxidant events (fig. 1).

ROS are highly reactive and, when generated close to cell membranes, deplete intracellular GSH and oxidize membrane phospholipids (lipid peroxidation), which may continue in a chain reaction. Thus, a single ·OH can result in the formation of many molecules of lipid hydroperoxide in the cell membrane, which may severely disrupt its function and may lead to cell death, or to damage of deoxyribonucleic acid (DNA) in alveolar epithelial cells [35]. ROS and reactive nitrogen species (RNS) also act on certain amino acids in proteins (*e.g.* enzymes, kinases) such as methionine, tyrosine and cysteine, profoundly altering the function of these proteins in inflammatory lung diseases [36]. Many of the effects of oxidants in airways may be mediated by the secondary release of inflammatory lipid mediators such as 4-hydroxy-2-nonenal, which is known to induce various cellular events

Table 1. – Epithelial lining fluid reduced glutathione (GSH) concentration in inflammatory lung diseases

	GSH μM		[Ref.]
	Controls	Patients	
Controls (nonsmokers)	339±112	–	[24]
Smokers	544±97.6	–	[24]
Idiopathic pulmonary fibrosis	429±34	97±18	[16]
Acute respiratory distress syndrome	651±103.1	31.5±8.4	[18]
Lung allograft	302.6±40.8	94.0±9.7	[20]
Cystic fibrosis	257±21	78±13	[19]
HIV-seropositive	245±12	170±23	[21]
Asthma	23.3±3*	36.5±9.4*	[22]

*: GSH plus oxidized glutathione (in μM·mg protein⁻¹). HIV: human immunodeficiency virus.

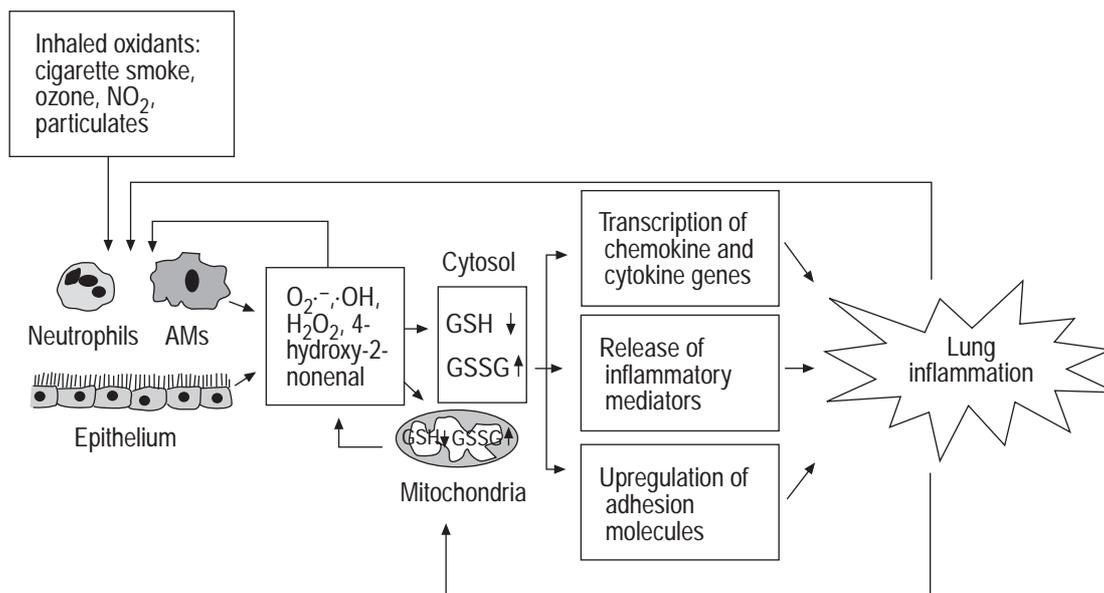


Fig. 1. – Mechanisms of oxidant-mediated lung inflammation. The inflammatory response is mediated by oxidants which are inhaled and/or released by the activated neutrophils, alveolar macrophages (AMs) and epithelial cells leading to depletion of the antioxidant reduced glutathione (GSH). Activation of transcription of the pro-inflammatory cytokine and chemokine genes, upregulation of adhesion molecules and increased release of pro-inflammatory mediators are involved in the inflammatory responses. GSSG: oxidized glutathione; ↓ : decrease; ↑ : increase.

such as proliferation and activation of signalling pathways [37].

Inhaled oxidants in lung inflammation

Inhaled environmental oxidants exacerbate the underlying inflammation in inflammatory lung diseases. Ozone is a potent oxidant, which causes cellular damage by lipid peroxidation as well as loss of functional groups on biomolecules. Inhalation of ozone may lead to an increase in neutrophil numbers, increased airway responsiveness and reduced pulmonary function in normal subjects [38]. This has been linked to neutrophil infiltration into the airway epithelium [39]. Cigarette smoking, another environmental hazard, also delivers oxidants and free radicals to the lungs. Cigarette smoke contains many oxidants and free radicals, both in the gas and the tar phase [40], and causes sequestration of neutrophils into the pulmonary microcirculation and accumulation of macrophages in respiratory bronchioles [41], with the potential to release oxidants [1, 41, 42]. It has been shown recently that the effect of cigarette smoke on acute increases in airway resistance and constriction occurs *via* a direct oxidant-mediated mechanism [43]. The release of ROS from activated neutrophils in the pulmonary microcirculation has been implicated as a contributor to the inflammatory responses in lung diseases [1, 31]. Nitrogen dioxide and sulphur dioxide are other inhaled air pollutant oxidants, which may alter lung function by the release of reactive electrophiles and the generation of oxidants [44–47]. Inhaled oxidants generated from air pollution particulates (particles with a 50% cut-off aerodynamic diameter of 10 μm (PM₁₀)) are also associated with the release of inflammatory cytokines by airway epithelial cells [48].

Activation of redox-sensitive transcription factors

Nuclear factor- κB

Oxidants, either inhaled or produced by inflammatory cells, are directly implicated in the inflammatory responses in lung cells *via* signalling mechanisms. Transcription factors such as NF- κB and AP-1, which are redox-sensitive [25, 49], have been shown to be activated in epithelial cells and inflammatory cells during oxidative stress/inflammation, leading to the upregulation of a number of pro-inflammatory genes [25]. Maintenance of a high intracellular GSH/GSSG ratio (>90%) minimizes accumulation of disulphides and provides a reducing environment within the cell. However, if oxidant or other environmental stress alters this ratio, this shift in the GSH/GSSG redox buffer influences a variety of cellular signalling processes, such as activation of the transcription factors AP-1 and NF- κB . Oxidative stress including the presence of lipid peroxidation products [50] or depletion of GSH and subsequent increases in cytosolic GSSG in response to oxidative stress causes rapid ubiquitination and phosphorylation and thus subsequent degradation of the inhibitor of NF- κB (I κB), which is a critical step for NF- κB activation [51, 52]. Under reducing conditions, such as an increase in intracellular GSH following treatment with *N*-acetyl-L-cysteine (NAC), the phosphorylation of serine groups on I κB - α following TNF- α treatment is inhibited, leading to the down-regulation of NF- κB in endothelial cells (fig. 2) [53].

NF- κB regulates the expression of many genes involved in inflammation whose products mediate inflammatory responses in the lungs such as inducible nitric oxide synthase, the pro-inflammatory cytokines IL-1 β , TNF- α and IL-6, the chemokine IL-8, E-selectin, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and granulocyte-macrophage colony-stimulating factor [4, 54, 55]. In many inflammatory

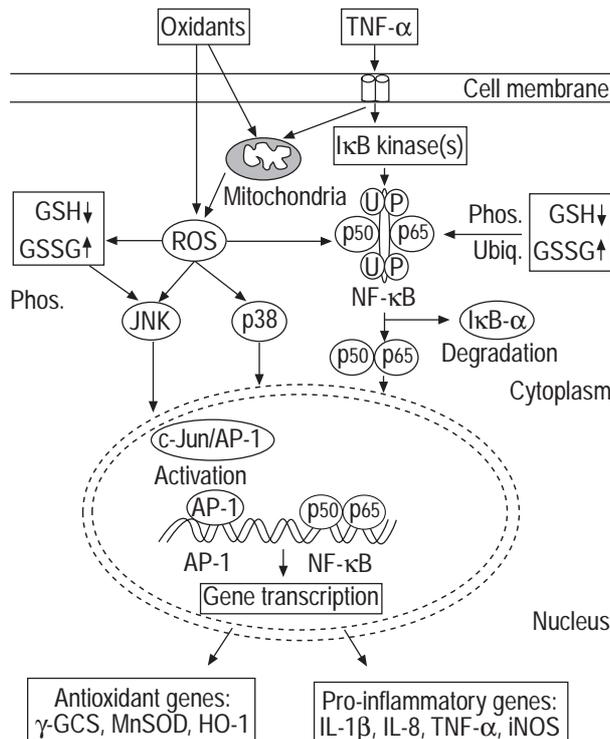


Fig. 2. – Model of the mechanism of nuclear factor- κ B (NF- κ B) and activation protein-1 (AP-1) activation leading to gene transcription in lung epithelial cells. Tumour necrosis factor- α (TNF- α)/oxidants act on mitochondria to generate reactive oxygen species (ROS), which are involved in the activation of NF- κ B and AP-1. Activation of NF- κ B involves the phosphorylation, ubiquitination and subsequent proteolytic degradation of the inhibitor of NF- κ B (I κ B). Free NF- κ B then translocates into the nucleus and binds to its consensus sites. The intracellular ratio of reduced glutathione (GSH)/oxidized glutathione (GSSG) can modulate AP-1 and NF- κ B activation. Similarly, either c-Jun/c-Jun (homodimer) or c-Fos/c-Jun (heterodimer) is activated by phosphorylation of the c-Jun N-terminal protein kinase (JNK), leading to the activation AP-1, which binds to its tetradecanoylphorbol-13-acetate response element (TRE) consensus regions. Activation of NF- κ B/AP-1 leads to the co-ordinate expression of antioxidant protective and pro-inflammatory genes. γ -GCS: γ -glutamylcysteine synthetase; MnSOD: manganese superoxide dismutase; HO-1: haemoxygenase-1; IL: interleukin; iNOS: inducible nitric oxide synthase; \downarrow : decrease; \uparrow : increase. Phos/P: phosphorylation; Ubiq/U: ubiquitination; p38: p38 kinase; p30, p65: NF- κ B subunits.

lung diseases such as IPF, ARDS, CF and HIV, depletion of intracellular GSH levels or increased GSSG levels are present concomitant with the induction of inflammatory mediators and chemotactic cytokines [8]. This suggests that the intracellular redox state (GSH/GSSG levels) of the cell may play a key role in the regulation and potentiation of the inflammatory responses in lung cells.

Activator protein-1

AP-1 is composed mainly of the Jun and Fos gene products, which form homodimeric (Jun/Jun) or heterodimeric (Jun/Fos) complexes. DNA-binding of the Fos/Jun heterodimer is increased by the reduction of a single conserved cysteine in the DNA-binding domain of each of the proteins [56]. Antioxidants such as NAC increase unstimulated and 12-*O*-tetradecanoyl-phorbol-13-acetate

(TPA)-stimulated AP-1 DNA-binding and transactivation in HeLa cells [57]. This may be due to conservation of the cysteine (sulphydryl) residue required for the nuclear AP-1 DNA binding. The DNA-binding of AP-1 can be enhanced by thioredoxin as well as the nuclear redox protein, Ref-1, and inhibited by GSSG in many cell types, suggesting that disulphide bond formation by cysteine residues inhibits AP-1 DNA-binding [58, 59]. By contrast, oxidative stress imposed by H₂O₂ treatment, depletion of intracellular GSH using DL-buthionine-(S,R)-sulphoximine (BSO) or an increase in the ratio GSH/GSSG by diamide treatment of the liver cell line HepG2 also stimulates AP-1 binding [60]. This suggests that another mechanism besides direct protein/DNA binding such as redox-sensitive signalling pathways are involved in the regulation of AP-1 activation. This supports work by WILHELM *et al.* [61] demonstrating that perturbation of cellular thiol redox status provides a signal for AP-1 activation by the induction of stress-activated signal transduction pathways by c-Jun N-terminal protein kinase (JNK) and p38 kinase. Moreover, as both oxidants and antioxidants stimulate AP-1, differences in biological responses to these agents are likely to be related to the extent of AP-1 activation and the distinct AP-1 subunits which are upregulated, and hence the response which is provided, since different AP-1 dimers can either stimulate or repress gene expression. In addition, activation of redox-sensitive JNK and p38 by pro-inflammatory cytokines, such as TNF- α and IL-1, leads to the induction of genes for cytokines, chemokines and various pro-inflammatory mediators, which play an important role in the inflammatory response [5, 62, 63].

Glutathione synthesis and its redox recycling

The synthesis of glutathione requires the presence of two enzymes and the amino acids, glycine, cysteine and glutamic acid, with cysteine being the rate-limiting substrate. The tripeptide GSH is formed by the consecutive actions of γ -GCS and glutathione synthetase [64].

In general, the activity of γ -GCS determines the rate of glutathione synthesis. The reaction, catalysed by γ -GCS is feedback-inhibited by GSH [64]. The mammalian γ -GCS holoenzyme is a heterodimer consisting of γ -GCS-HS (73 kDa) and γ -GCS-LS (30 kDa) [65]. Although γ -GCS-HS contains all of the catalytic activity, γ -GCS activity can be modulated by the association of γ -GCS-HS with the regulatory γ -GCS-LS [65]. The regulatory properties of γ -GCS-LS have been proposed to be mediated by a disulphide bridge between the subunits that would allow conformational changes in the active site depending on the oxidative state of the cell [65]. This implies that potential for increasing the rate of GSH synthesis exists under conditions of GSH depletion.

The GSH redox system is crucial in maintaining intracellular GSH/GSSG homeostasis, which is critical to normal cellular physiological processes, and represents one of the most important antioxidant defence systems in lung cells [64]. This system uses GSH as a substrate in the detoxification of peroxides such as H₂O₂ and lipid peroxides, a reaction which involves glutathione peroxidase. This reaction generates GSSG which is then reduced to

GSH by glutathione reductase in a reaction requiring the hexose monophosphate shunt pathway utilizing NADPH.

Regulation of glutathione and γ -glutamylcysteine synthetase

At transcriptional level

Identification and characterization of the types of diverse stimuli that act as potent inducers of γ -GCS should aid in the development of effective pharmacological strategies for antioxidant treatment involving GSH regulation in inflammatory lung diseases. To this end, several studies have been directed towards understanding and elucidating the molecular mechanisms of GSH synthesis and regulation in type II alveolar epithelial cells in response to various environmental oxidants, antioxidants and inflammatory stimuli (table 2). The authors' group and other investigators have reported that the promoter (5'-flanking) region of the human γ -GCS-HS gene is regulated by a putative c-Jun homodimer (AP-1) binding site

Table 2. — Inducers of glutathione and γ -glutamylcysteine synthetase

Agent	[Ref.]
Oxidants	
Hydrogen peroxide	[30, 70]
Menadione	[30, 70–72]
Cigarette smoke	[13, 29]
Dimethyl naphthoquinone	[73, 74]
Xanthine/xanthine oxidase	[71]
<i>t</i> -butyl hydroperoxide and hydroquinone	[27, 67, 68, 75]
4-Hydroxy-2-nonenal	[37, 76]
Ozone	[77]
Hyperoxia	[71]
Phenolic antioxidants	
β -Naphthoflavone	[28, 78]
Apocynin	[79]
Butylated hydroxyanisole	[80]
Butylated hydroxytoluene	[81]
Pyrrolidinedithiocarbamate	[82, 83]
5, 10 dihydroindeno(1,2- <i>b</i>) indole	[84]
Cytokines	
TNF- α	[85, 86]
IL-1 β	[87]
Nitric oxide donors	
Nitric oxide	[88]
DETA NONOate	[89]
<i>S</i> -nitrosopenicillamine	[90]
Radiation	
Ionizing radiations	[91, 92]
Metals	
Selenium	[93]
Iron	[94]
Cadmium	[95]
Mercury	[96]
Chemotherapeutic agents	
Cisplatin	[66, 97]
Melphalan	[98]
Growth factors	
Nerve growth factor	[99]
Acid fibroblast growth factor	[100]
Others	
Heat shock	[101]
Oxidized low density lipoprotein	[69]

TNF- α : tumour necrosis factor- α IL: interleukin.

[30, 66–69]. The sequence for this binding site is located in the proximal region of the γ -GCS-HS TATA box in various cell lines, including human alveolar epi-thelial cells [30, 67, 68].

MULCAHY and coworkers [28, 78], however, have reported a distal ARE containing an embedded TPA-responsive element (TRE) and an electrophile responsive element (or its functional equivalent ARE), which play a key role in the regulation of the γ -GCS-HS and γ -GCS-LS, respectively, in response to a planar aromatic xenobiotic, the phenolic antioxidant β -naphthoflavone, specifically in HepG2 cells. They also showed that the internal AP-1 site is important for the constitutive expression of the γ -GCS-LS gene [78]. However, recently, GALLOWAY and coworkers [27, 75] were unable to demonstrate a role for ARE in the induction of γ -GCS-LS by oxidants such as *t*-butyl hydroquinone in HepG2 cells. They suggested that an AP-1 site was the critical element for the basal regulation of this subunit. Therefore, it is likely that the expression of the γ -GCS subunit genes is regulated by different regulatory signals in response to diverse stimuli in specific cells.

Exposure to phenolic antioxidants such as dietary 2(3)-*t*-butyl-4-hydroxyanisole and butylated hydroxytoluene as well as the synthetic indolic antioxidant 5,10-dihydroindeno(1,2-*b*) indole and pyrrolidine dithiocarbamate (PDTTC), a sulphhydryl-modifying antioxidant compound, upregulate γ -GCS-HS and γ -GCS-LS in human endothelial cells and other cell lines [75, 80–84]. The plant-derived phenolic antioxidant apocynin (4-hydroxy-3-methoxyacetophenone) also induces GSH synthesis in human alveolar epithelial cells [79]. These effects of phenolic antioxidants are associated with AP-1 transactivation [57, 60, 102]. 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 levels.

A role for NF- κ B in the modulation of γ -GCS-HS gene expression has also been suggested [91, 103, 104]. It has been shown that blocking the activation of NF- κ B, which is present at the transcriptional site of the γ -GCS-HS promoter, by various strategies prevented oxidant/cytokine-induced increase in γ -GCS-HS transcription in mouse endothelial cells and hepatocytes [91, 103]. However, mutation and deletion techniques applied to the γ -GCS-HS promoter region have ruled out the possible involvement of NF- κ B in the transcriptional upregulation of the γ -GCS-HS gene in alveolar epithelial cells and other cell lines in response to TNF- α and oxidative stress [29, 30, 68, 85–87].

At post-transcriptional and translational levels

Regulation of GSH and γ -GCS has also been described at the post-transcriptional and pretranslational levels in rat liver *in vivo* and in other cells [105, 106]. Various inflammatory agents such as cyclic adenosine monophosphate (cAMP) and intracellular calcium, which are released during inflammation, may inhibit GSH synthesis at the translational level (table 3) [107]. It has been shown that γ -GCS activity is inhibited by agonists of various signal transduction pathways in rat hepatocytes [107], suggesting a role for signalling mechanisms in the

Table 3. – Inhibitors of glutathione synthesis

Agent	[Ref.]
Glucocorticoid (dexamethasone)	[71, 86]
Transforming growth factor- β	[109, 110]
Cyclic adenosine monophosphate	[107, 108]
Cytosolic free calcium	[107]
Insulin and hydrocortisone	[107]
Prostaglandin E ₂	[111]

regulation of GSH levels. Lu *et al.* [107] 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 signalling pathways, these investigators determined that hormone-specific inhibition of GSH synthesis was mediated by the activation of protein kinase A, protein kinase C and Ca²⁺/calmodulin-dependent kinase II. This inhibition of GSH synthesis correlated with the direct phosphorylation of γ -GCS-HS on serine and threonine residues which was dependent on the concentration of Mg²⁺. 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* [108]. Thus, phosphorylation/dephosphorylation may regulate γ -GCS activity [108] and may provide a mechanism for altering GSH levels in lung cells during oxidative stress.

Role of γ -glutamyl transpeptidase in the regulation of glutathione levels in lungs

Modulation of γ -glutamyl transpeptidase (γ -GT) may be another avenue for the regulation of intracellular GSH levels in lung cells. γ -GT cleaves extracellular GSH into its constituent amino acids and leads to the resynthesis of intracellular GSH rather than direct intact cellular GSH uptake [112]. The enzyme γ -GT is a plasma membrane enzyme, with its active site directed toward the outside of the cell, and is present in lung epithelial cells. This enzyme breaks the γ -glutamyl bond of γ -glutamyl-cysteinyl-glycine [113]. 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. The lung epithelium has been shown to contain high levels of γ -GT activity and utilizes extracellular GSH from the alveolar lining fluid [114, 115]. Hence most plasma GSH is catabolized by the enzyme γ -GT in lungs [114, 115]. 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 [113, 115, 116].

In an animal model, rats exposed to hyperoxia exhibited low γ -GT activity in ELF; this was associated with low ELF GSH levels [117]. γ -GT expression is increased in rat lung epithelial cells by oxidants such as menadione and *t*-butyl hydroquinone [118], suggesting that γ -GT might play a role in protection against oxidative stress. However, cigarette smoke condensate and oxidative stress had no effect on γ -GT activity in a human type II alveolar

epithelial cell lines (A549 cells) [71]. A 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. However, the role of the direct involvement of γ -GT in the lungs of smokers remains to be proven.

Cystine/cysteine transport and regulation of glutathione levels in lung cells

The rate-limiting step in the biosynthesis of GSH is the availability of cysteine as a substrate within the cell [64]. Cystine, an oxidized form of cysteine, is efficiently transported in 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 [119–122]. Intracellular transport of cystine is accompanied by the extracellular release of glutamate. Cysteine is also transported into cells by sodium-dependent amino acid transport systems (labelled as A or ASC) shared with glutamine and serine [123]. 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 analogues into cells against a concentration gradient [124–126]. These transport systems may increase intracellular GSH levels in lung cells, and might be one of the alternative mechanisms modulating intracellular GSH levels in lungs.

Various forms of oxidant stress and NO also increase the activity of membrane cystine and glutamate transport leading to increased GSH synthesis in lung cells [90, 122, 127, 128]. It has been clearly shown that the cystine uptake is the rate-limiting step for GSH synthesis in cultured lung cells, especially under conditions of oxidative stress [66, 129]. Oxidants (hyperoxia and H₂O₂), 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 sodium-independent inducible system specific to the intracellular transport of cystine and glutamate [130–133]. It has been shown that exposure of rats to hyperoxia results in increases in total lung GSH within 24 h [6, 134]. It is, therefore, possible that induction of cystine or cysteine transport could contribute to the increased GSH levels in lungs after exposure to hyperoxia [6, 134].

The regulation of cystine/glutamate transport is governed by the availability of extracellular cysteine or cystine as well as the extracellular redox state (which is, in part determined by extracellular GSH levels) [120, 135]. Treatment with reducing agents such as NAC or GSH increases intracellular GSH levels by making intracellular cysteine available and reducing cystine to cysteine in bovine pulmonary artery endothelial cells [121]. Furthermore, NAC increases intracellular GSH levels in bovine pulmonary artery endothelial cells even in the absence of cystine in the medium [135]. This suggests that a different transport mechanism independent of the X_c⁻ system may be involved in type II epithelial cells in increasing GSH levels in response to various stresses [125]. Nevertheless, this is one of the mechanisms whereby lung cells increase

intracellular GSH levels under various stresses (either oxidant stress or GSH depletion).

The levels of intracellular GSH are regulated, in part, by the rate of the bidirectional membrane transport system present in lung and liver cells [136, 137]. It is likely that this membrane transport system causes GSH to efflux to the lung ELF. The function of such a GSH transport system is influenced by the redox/thiol status of the cell, the membrane potential and presence of cations in the extracellular environment [138, 139]. GSH-related structural compounds, such as glutathione-S-conjugates and GSH ethyl ester, inhibit cellular GSH uptake or influx [137, 138]. Furthermore, a more oxidized extracellular environment stimulates cells to retain GSH, whereas a more reduced extracellular state facilitates GSH efflux [137, 140]. However, these effects are in direct contrast with the situation in lungs *in vivo*, since the increased oxidant burden imposed by smoking and endogenous oxidative stress during inflammation could cause lung cells to retain GSH, rather than release it into the ELF. This mechanism is difficult to explain in the presence of such a bidirectional GSH transporter in the lung. Thus the mechanisms that determine the levels of GSH in the lung ELF using the bidirectional transporter are not fully understood.

Regulation of glutathione in oxidant-mediated susceptibility/tolerance

Effect of pro- and anti-inflammatory mediators

TNF- α is a ubiquitous pro-inflammatory 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 defence mechanisms including antioxidant levels [141]. TNF- α is an important inflammatory mediator in COPD and ARDS and is present in increased amounts in the bronchoalveolar lavage fluid (BALF) and sputum of COPD patients [142]. Recently, various investigators [86, 143] have shown rapid depletion of intracellular GSH by TNF- α exposure in epithelial and endothelial cells *in vitro*, due to oxidation of GSH to GSSG. This is followed by a rebound increase in GSH levels in epithelial and endothelial cells as an adaptive response to oxidant stress, occurring as a result of upregulation of the γ -GCS-HS and activation of AP-1 [85, 87]. Similarly, exposure of fibroblasts to prostaglandin E₂, an inflammatory mediator capable of regulating fibroblast cell proliferation and matrix protein production, resulted in decreased GSH synthesis [111]. Furthermore, GSH concentrations in peripheral blood lymphocytes may be decreased as a result of lung inflammation and may be inversely correlated with lung function in patients with CF [144]. Thus oxidative stress imposed by inflammatory mediators may acutely deplete GSH during inflammation and render cells susceptible to the amplification of inflammatory responses.

Glucocorticoids, such as dexamethasone, are widely used as anti-inflammatory agents in various inflammatory lung diseases. Airway epithelium is one of the most important targets for inhaled glucocorticoids in lung diseases [145]. Exposure of lung epithelial A549 cells to de-

xamethasone decreases both basal and stimulated GSH levels (TNF- α -treated) in these cells [71, 86]. Dexamethasone also decreases γ -GCS-HS gene expression in alveolar epithelial cells *in vitro* by a transcriptional mechanism involving inhibition of the AP-1 transcription factor [86]. Thus it is possible that the use of dexamethasone in patients with inflammatory lung diseases may prevent synthesis of the protective antioxidant GSH.

Effects of oxidants

Exposure of alveolar epithelial cells *in vitro* to oxidants, such as H₂O₂, hyperoxia and redox recycling compounds like menadione, causes an initial depletion of GSH, associated with increased formation of GSSG, followed by a rebound increase in GSH levels at 24 h [29, 70–72]. This is concomitant with increased expression of messenger ribonucleic acid (mRNA) for the γ -GCS gene [30, 70, 72]. Epithelial cells also take up glutathione by a redox-dependent mechanism [137]. This transport system may act to regulate intracellular glutathione depending on the presence of reduced thiols or disulphides in the extracellular environment [138]. Thus, the short-term effects of various oxidants and oxidant-generating systems appear to be to upregulate the gene for glutathione synthesis, possibly providing a protective/adaptive mechanism against subsequent oxidative stress (table 2).

Oxidative stress produced by ozone [77], xanthine/xanthine oxidase [71], lipid peroxidation products (4-hydroxy-2-nonenal) [76], ionizing radiation [91, 92], hypoxia [146] and heat shock [101] all lead to short-term falls in intracellular GSH levels, followed by increases in GSH levels or upregulation of γ -GCS-HS mRNA in alveolar epithelial cells, endothelial cells *in vitro* and other cell types as well as *in vivo* in rats. NO and its donors, such as S-nitrosopencillamine or DetaNONOate, also cause transient depletion of GSH followed by induction of GSH synthesis by enhanced expression of the γ -GCS-HS and γ -GCS-LS in rat aortic vascular smooth muscle cells [90], pulmonary fibroblasts [89] and bovine aortic endothelial cells [88]. The increase in GSH levels caused by NO donors is a further potential mechanism for protecting cells against oxidative stress and subsequent inflammation. Oxidative stress imposed by heavy metals such as selenium [93], iron [94], methyl mercury [96], and cadmium [95] also induce GSH synthesis in various organs in both rats and mice. Other cytotoxic compounds that act through the generation of ROS such as the chemotherapeutic agents cisplatin [66, 97] and melphalan [98] also increase GSH synthesis in cancer cell lines. These drugs may also coinduce the multidrug resistance-associated protein (MRP) gene and the adenosine triphosphate-dependent transporter, glutathione-conjugate (GS-X) pump, in lung cells [147–150]. The functional role of MRP coexpression in lung cells is currently unknown. It is possible that the coexpression of MRP and GS-X is related to protection against the inflammatory response and detoxification of xenobiotics and endogenous cysteinyl leukotrienes [151]. The induction of GSH synthesis may be associated with activation of mitogen-activated protein kinases, particularly c-JNK, in response to oxidants, heavy metals and NO [37]. 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 be a potential mechanism for enhancing cellular GSH synthesis, and so developing cellular tolerance to oxidative stress [28, 73]. Exposure to sublethal doses of oxidants and oxidant-generating systems may initiate an adaptive intracellular antioxidant response, thus protecting cells from subsequent exposures to oxidant stresses [74, 152]. It is possible that the GSH synthesis and tolerance mechanism which occurs in response to various stimuli described in various cells may differ in lung cells.

Effect of growth factors

Transforming growth factor (TGF)- β 1 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 cell [153]. Recent studies have shown increased expression of TGF- β 1 in bronchiolar and alveolar epithelium in IPF and COPD patients, and higher levels in the BALF of atopic asthmatics as compared to healthy subjects [154, 155]. TGF- β 1 also downregulates γ -GCS-HS mRNA and glutathione synthesis in human alveolar epithelial cells and pulmonary artery endothelial cells *in vitro* [109, 110]. Interestingly, recent studies by FACTOR *et al.* [156] showed decreased glutathione synthesis in a TGF transgenic (overexpression) mouse model and increased susceptibility to oxidant-mediated injury. Various workers have shown that γ -GCS-HS mRNA expression is under the control of the AP-1 transcription factor [31, 67, 68, 86], and that TGF- β 1 may decrease γ -GCS-HS gene expression *via* an AP-1 mechanism [157]. Thus higher levels of TGF- β 1 may downregulate glutathione synthesis in the lungs of patients with inflammatory diseases such as IPF and COPD. Moreover, decreased GSH levels may also have direct functional consequences. *In vitro* studies showed that GSH (in the concentration range normally found in ELF) suppressed fibroblast proliferation [158]. The relevance of GSH regulation and subsequent tolerance/susceptibility in lung epithelial cells in response to pro-/anti-inflammatory mediators and/or oxidants under conditions of chronic inflammation *in vivo* is not known.

Differential regulation of glutathione and γ -GCS gene expression have been demonstrated by other growth factors such as nerve growth factor [99] and plating cells at a low cell density [103, 159, 160]. Intracellular GSH levels have also been shown to be elevated in response to mitogenic stimulation as cells exit from their quiescent state and conditions that lead to cellular transformation [161–165]. Extracellular acid fibroblast growth factor (FGF-1) has been demonstrated to cause transformation and aggressive cell growth in murine embryonic fibroblasts. Recent data from CHOI *et al.* [100] have shown that expression of a chimeric human FGF-1 gene containing a signal peptide sequence for secretion (hst/KS0FGF-1) in an embryonic fibroblast cell line caused increased gene expression of both γ -GCS subunits associated with increased γ -GCS activity without elevation of intracellular GSH levels [100]. They suggested that an increase in GSH content *per se* is not required for

altered cell growth though increased expression of γ -GCS and γ -GCS activity is associated with a common response to growth factors.

Role of γ -glutamylcysteine synthetase in genetic susceptibility to disease

Recent studies have shown that GSH regulation/the GSH redox system might be one of the factors involved in genetic susceptibility to oxidant/pollutant-mediated lung cell damage. For example genetic susceptibility to cigarette smoke has been suggested as a risk factor for the development of COPD [166, 167]. Furthermore, it has been shown that polymorphic expression of several different xenogenes, including those encoding cytochrome P-450 1A1 (CYP1A1), glutathione-S-transferases (GSTs) (GST-M1 and GST-pi) and microsomal epoxide hydrolase, as well as pro-inflammatory genes such as TNF- α , is associated with an increased risk of chronic inflammatory lung diseases [168–172] and inflammatory response [173, 174]. The expression of these genes is directly or indirectly related to the modulation of enzymes of the glutathione redox system and γ -GCS [85–87, 102, 166]. Variations in the expression of the γ -GCS gene in humans may represent a new susceptibility factor in 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 [175]. Genetic analysis of 50 unrelated Caucasians identified three alleles as follows: A1 (nine repeats, 35% frequency), A2 (eight repeats, 11% frequency), and A3 (seven 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. Depletion of GSH levels has been associated with a genetic polymorphism and deficiency of γ -GCS activity in a patient with haemolytic anaemia [176]. Genetic analysis revealed that the γ -GCS polymorphism was associated with an A to T mutation/transversion at nucleotide 1109 that predicted substitution of histidine with leucine at amino acid 370, a diallelic polymorphism in nucleotide +206 of an intron and another polymorphism that consisted of a duplication of CAGC at complementary DNA (cDNA) nucleotides 1972–1975 in the 3'-untranslated region of the γ -GCS catalytic subunit. This mutation was not present in the γ -GCS DNA of 38 healthy subjects [176]. The presence of such mutation and possibly associated genetic polymorphism in the coding and/or noncoding region of the γ -GCS catalytic/regulatory subunit might be associated with the depletion of GSH in the inflammatory response and susceptibility to chronic inflammatory diseases.

The γ -GCS subunit genes are located on separate chromosomes and expression of their mRNA varies considerably between different tissues [175, 177, 178]. Human γ -GCS-HS is located on chromosome 6 (6p12) and γ -GCS-LS on chromosome 1 (1p21) [177–179]. Genetic analysis reveals that a frequent deletion of the γ -GCS-LS chromosome, 1p22→p21, occurs in human malignant mesothelioma. This gene deletion is considered to predispose an individual to the development of mesothelioma [180]. Recent data have demonstrated that AP-1 and ARE, which are present in the promoter region of the

γ -GCS-HS and γ -GCS-LS genes, may be directly involved in the regulation of GSH in human cells [28, 30, 75, 78]. Within a population, it is likely that there will be variation (gene deletion or mutation) in the 5'-coding/noncoding regions of γ -GCS-HS and γ -GCS-LS genes. Future studies need to be directed towards understanding the nature of any polymorphisms that exist and whether any association exists between these polymorphisms and susceptibility to the development of inflammatory lung diseases such as COPD and IPF.

Role of glutathione in the regulation of pro-inflammatory and antioxidant protective genes

There is increasing evidence to suggest that many inflammatory lung diseases are associated with airway/airspace inflammation and/or oxidant/antioxidant imbalance leading to alteration in glutathione levels in the ELF. It is also well documented that many pro-inflammatory and antioxidant genes are regulated by a redox-dependent signalling mechanism in lung cells. Hence critical regulation of intracellular glutathione levels under oxidative stress and inflammation might determine the expression of pro-inflammatory and antioxidant genes. Therefore, it is likely that both redox GSH levels and the various forms of oxidative stress (ROS)/nitrosative stress would determine the regulation of specific genes for pro-inflammatory mediators and antioxidant enzymes.

Pro-inflammatory genes

Inflammatory mediators play a crucial role in chronic inflammatory processes and appear to determine the nature of the inflammatory response by directing the selective recruitment and activation of inflammatory cells and their perpetuation within the lungs. In *in vitro* studies, using macrophages and alveolar and bronchial epithelial cells, oxidants (ROS) have been shown to cause both the release of inflammatory mediators such as IL-8, IL-1 and NO and increased expression of pro-inflammatory genes *via* alteration in redox GSH-dependent mechanisms [181–183]. The genes for these inflammatory mediators are regulated by transcription factors such as NF- κ B. This critical transcription factor in the inflammatory response is redox-sensitive. It is also known that various forms of nitrosative stress (reactive nitrogen intermediates/RNS or NO donors) have an effect on intracellular lung GSH levels leading to the expression of various pro-inflammatory mediators such as TGF- β 1 and activation of nuclear enzymes such as poly (adenosine diphosphate-ribose) synthetase, which is involved in inflammation [184–186].

The modulation of intracellular thiol status not only quenches oxidants/free radicals but also buffers the antioxidant potential of the cell and detoxifies electrophilic compounds. Thiol antioxidants such as NAC and *N*-acetyln (lysine salt of *N*-acetyl-L-cysteine), which have potential as therapies in inflammatory diseases, have been shown, in *in vitro* and *in vivo* experiments, to block the release of these inflammatory mediators from epithelial cells and macrophages by a mechanism involving increasing intracellular GSH levels and decreasing NF- κ B activation [182, 183, 187].

Antioxidant protective genes

An important effect of oxidative stress and inflammation is the upregulation of protective antioxidant genes (fig. 3). Among the antioxidant enzymes, GSH and its redox enzymes appear to play an important protective role in the airspaces and intracellularly in epithelial cells. The protective role of GSH against the effects of cigarette smoke/oxidants has been demonstrated both *in vivo* in the rat and *in vitro* using monolayer cultures of alveolar epithelial cells [13, 14, 188]. Acute intratracheal instillation of cigarette smoke condensate in the rat and exposure of epithelial cell monolayers to cigarette smoke *in vitro* [13] lead to a profound decrease in GSH levels in BALF, and in the lungs of rats and epithelial cells. This is followed by a rebound adaptive increase in GSH levels and γ -GCS-HS mRNA expression in both rat lungs and epithelial cell lines [13, 189]. This finding is mirrored in humans, in whom GSH levels are elevated in ELF associated with increased expression of γ -GCS mRNA in the lungs of chronic cigarette smokers, whereas this is not the case in acute smoking compared to nonsmokers [23, 24, 190]. Thus oxidative stress, including that produced by cigarette smoking, causes upregulation of an important gene involved in the synthesis of GSH as an adaptive mechanism against subsequent oxidative stress. However, this adaptive response may not counteract the potential burden of pro-inflammatory mediators and oxidants released during inflammation.

A recent study has shown that expression of γ -GCS mRNA is elevated in smokers' lungs and that this is even more pronounced in smokers with COPD [190]. This implies that GSH synthesis might be upregulated (GSH levels were not studied) in the lungs of smokers with and without COPD. Similarly, rats exposed to cigarette smoke have shown increased expression of genes encoding manganese SOD (MnSOD), metallothionein and glutathione peroxidase (GPx) in bronchial epithelial cells, suggesting the importance of the antioxidant gene adaptive response against the injurious effects of cigarette smoke [191]. Important protective antioxidant genes such as those encoding MnSOD, γ -GCS-HS, haem oxygenase-1 (HO-1), GPx, thioredoxin reductase and metallothionein are induced by modulation of cellular GSH/GSSG

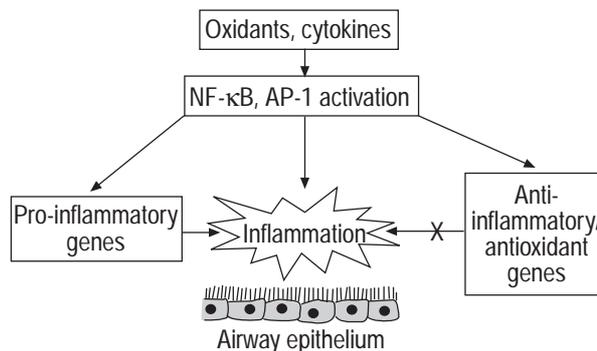


Fig. 3. – Activation of the transcription factors nuclear factor- κ B (NF- κ B) and activation protein-1 (AP-1) by oxidants and/or pro-inflammatory cytokines leads to the induction of both pro-inflammatory and anti-inflammatory/antioxidant genes in lung cells. Products of pro-inflammatory genes cause airway inflammation, which is inhibited (X) by anti-inflammatory and/or antioxidant genes.

levels in response to various oxidative stresses including hyperoxia and inflammatory mediators such as TNF- α and lipopolysaccharide in lung cells [85, 86, 141, 192, 193].

Thus oxidative/nitrosative stresses, including redox modulation, cause increased gene expression of pro-inflammatory genes *via* oxidant-mediated activation of transcription factors such as AP-1 and NF- κ B and also activation of stress response protective genes such as γ -GCS-HS, HO-1 and MnSOD in lungs. A balance may therefore exist between pro- and anti-inflammatory gene expression and the levels of GSH in response to oxidative stress and during inflammation, which may be critical to whether this leads to cell injury or protection against the injurious effects of inflammation (fig. 4). Knowledge of the molecular mechanisms that sequentially regulate this battery of genes in relation to GSH levels in lung cells may open new therapeutic avenues in the modulation of inflammatory responses in lung diseases.

Protective role of glutathione in oxidant and free radical-mediated lung injury

Alveolar epithelial cells are important in maintaining the integrity and fluid balance of the lungs. The epithelium lining the airways and alveoli has a protective barrier function. The lower respiratory tract is sensitive to injury from inhaled and locally produced oxidants. In response to injury, the epithelium loses its selective permeability and becomes more permeable to the movement of water, ions and macromolecules. Increased epithelial permeability is one of the earliest events in lung injury and may enhance the inflammatory process by allowing easier access for

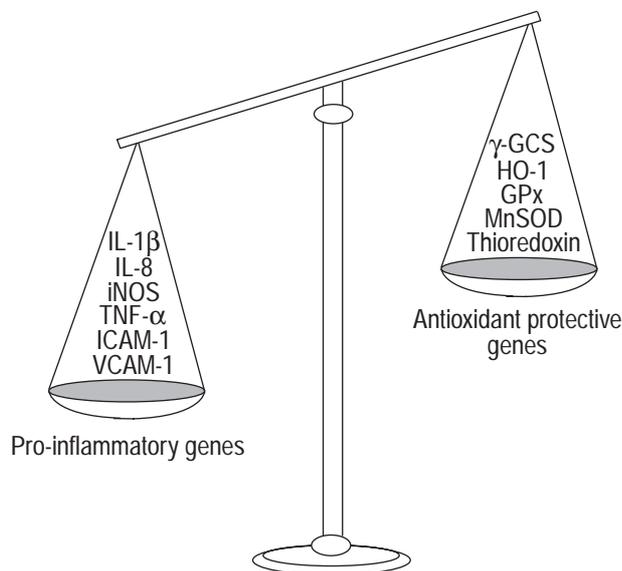


Fig. 4. – Pro-inflammatory and antioxidant protective gene imbalance in inflammation. In inflammation, the balance appears to be tipped in favour of increased pro-inflammatory mediators, due to either release of inflammatory mediators or amplification of the pro-inflammatory effects. Induction of antioxidant protective genes may be a delayed response and decline sharply. IL: interleukin; iNOS: inducible nitric oxide synthase; TNF- α : tumour necrosis factor- α ; ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule-1; γ -GCS: γ -glutamylcysteine synthetase; HO-1: haem oxygenase-1; GPx: glutathione peroxidase; MnSOD: manganese superoxide dismutase.

inflammatory and injurious mediators between the blood, interstitium and alveolar space.

Cigarette smoke/air particulate-mediated lung injury

Alveolar cells are normally covered in a thin protective layer of epithelial fluid, which is rich in antioxidants such as GSH [194]. It has been reported that incubation with extracellular GSH and increasing intracellular GSH levels protect against oxidant stress in alveolar type II cells [7, 195]. In addition, extracellular GPx, which has been described recently [196], is secreted into ELF by alveolar epithelial cells and macrophages and may provide further defence against oxidants [196]. Following acute inflammation and oxidative stress, the ELF may become depleted of antioxidants such as GSH, increasing the potential for damage to the underlying epithelial cells. Both *in vivo* and *in vitro* in monolayers of cultured epithelial cells, this decrease in GSH was associated with an increase in airspace epithelial permeability [13, 14, 188]. Decreasing GSH levels in both these *in vivo* and *in vitro* models using the γ -GCS inhibitor BSO produces increased epithelial permeability [13]. NISHIKAWA *et al.* [197] recently demonstrated that acute cigarette smoke exposure, in guinea-pigs, produced neutrophil influx into the airways associated with NF- κ B activation and IL-8 mRNA expression in alveolar macrophages. This may be due to GSH depletion of lung and alveolar macrophages by cigarette smoke. Furthermore, LI *et al.* [198] reported that instillation of air particulate matter (PM10) into the lungs of rats caused inflammation, decreases in lung GSH levels and increases in epithelial permeability. Oxidative stress and inflammation in response to air particulates have been shown to be prevented by NAC treatment in alveolar epithelial cells [48, 199]. Similarly, other air pollution gases such as SO₂ and NO₂ produce an inflammatory response and alveolar permeability *via* depletion of lung GSH levels in rats and A549 epithelial cells [44, 200, 201]. The decrease in GSH levels was associated with inhibition of several enzymes involved in the GSH redox system and glutathione synthesis, and the production of lipid peroxidation products in rat lungs and human alveolar epithelial cells [46, 200, 202, 203]. These studies suggest that GSH plays a critical role in maintaining epithelial membrane integrity and may protect epithelial cells against the inflammatory response produced by either inhaled or endogenous oxidants/free radicals. Furthermore, LINDEN and coworkers [204, 205] demonstrated that airway obstruction, measured by means of the forced expiratory volume in one second (FEV₁) in patients with COPD, correlated significantly with the concentration of GSH in BALF; the higher the BALF GSH, the lower the FEV₁. It may be that the BALF GSH levels were influenced by the recent smoking history of these patients [24].

Against inflammatory events in lungs

Neutrophil/endothelial interactions are events necessary for the progression of inflammatory responses in lung diseases. Recently, it has been shown that changes in endothelial cell GSH/GSSG ratio produce expression of different adhesion molecules on the cell surface, which are

associated with enhanced neutrophil/endothelial adhesion [206]. Agents that cause oxidation of GSH led to increases in neutrophil adhesion to endothelial cells *via* upregulation of ICAM-1 and VCAM-1 [207, 208], and increasing intracellular thiol concentrations with NAC attenuated the oxidant- or cytokine-mediated neutrophil adhesion to endothelial cells [208]. Therefore, a change in intracellular GSH redox balance may be an important mechanism in neutrophil adhesion, which is involved in chronic lung inflammation.

Modulation of growth factor receptors and altered cellular signalling is proposed to occur through a redox-mediated mechanism in inflammatory and lung cells. Tyrosine phosphorylation of the epidermal growth factor (EGF) receptor in lung epithelial cells by H_2O_2 is thought to influence inflammatory processes in lungs [209]. H_2O_2 is known to induce apoptosis in many cells including epithelial cells, and this response is inhibited by glutathione [3, 9, 210, 211]. In addition, a decrease in intracellular GSH levels in alveolar macrophages caused by oxidants, hyperoxia and cigarette smoke produces downregulation of vascular endothelial growth factor (VEGF) and its functional receptor [212, 213]. Downregulation of VEGF and its receptor may be associated with apoptosis, which may be linked in the pathogenesis of inflammatory lung diseases such as emphysema and COPD. GSH and other thiols such as NAC inhibit TNF- α -induced sphingomyelin hydrolysis, ceramide generation and programmed cell death (apoptosis), suggesting that GSH has antiapoptotic properties through its ability to detoxify oxidants and free radicals [214].

HO-1 is a member of the heat shock family of proteins, which plays an important role in inflammation. A role for GSH in the regulation of heat shock factor and activation of heat shock protein has been suggested [215]. The intracellular levels of GSH in fibroblasts modulate oxidant-induced expression of HO-1 [216]. This effect was due to the direct involvement of AP-1 (Jun/Jun) binding [192]. Similarly, metal-induced expression of the heat shock protein gene *hsp72* is attenuated by glutathione, implying a protective role of GSH in acute inflammation [217]. Hence, it is clear that maintenance of intracellular GSH levels is important in the control of inflammatory responses in lungs involving heat shock proteins.

Oxidant-mediated mitochondrial damage

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-catalysed reactions. Mitochondria contain 15–20% of the total cellular GSH. The mitochondrial GSH pool is derived solely from the activity of a mitochondrial transporter that translocates GSH from the cytosol into the mitochondrial matrix, as mitochondria do not possess the enzymes γ -GCS or γ -GT [218]. Mitochondrial GSH may also be susceptible to the oxidative stress imposed by TNF- α , and inhaled or endogenous oxidants in human lungs [10, 219]. TNF- α is known to deplete cytosolic GSH levels transiently in lung epithelial and endothelial cells [86, 143, 220]. This depletion by TNF- α is thought to be due to oxidative stress from mitochondrial leakage of $O_2^{\cdot-}$ *via* the electron transport chain [143, 221]. Oxidation of GSH is associ-

ated with activation of NF- κ B and damage to mitochondrial DNA leading to apoptosis in fibroblasts *in vitro* and a decline in lung function in smokers [219, 221, 222]. It is likely that mitochondrial GSH plays a key role in maintaining cellular antioxidant defence systems, and thus cell integrity and function, under various conditions of oxidative stress [221, 223]. CHEN *et al.* [220] have recently demonstrated that depletion of mitochondrial GSH in human umbilical vein endothelial cells (HUVECs) increased TNF- α -induced adhesion molecule (VCAM-1) expression but not ICAM-1 expression and mononuclear leukocyte adhesion in HUVECs, suggesting that mitochondrial GSH is involved in endothelial cell function [220]. Recent studies have shown that gene delivery of glutathione reductase to mitochondria and overexpression of GPx in various cell lines provided protection against oxidative stress [224, 225]. This finding demonstrates the importance of mitochondrial GSH homeostasis in the regulation of cell function. It is possible that an imbalance in mitochondrial GSH redox status may help to perpetuate inflammation in lung cells.

Oxidant-mediated extracellular matrix remodelling

Intracellular redox GSH levels have been shown to be involved in the remodelling of ECM during oxidant-mediated lung injury [226, 227]. This is supported by two observations: 1) oxidant-induced lung injury was attenuated by the synthetic matrix metalloproteinase (MMP) inhibitor British Biotech (BB)-3103 [228]; and 2) depletion of intracellular GSH was associated with activation of MMPs, thereby increasing degradation of the alveolar ECM in lungs [227]. This breakdown of lung ECM by MMP-9 and MMP-2 activation was blocked by increasing lung glutathione levels [227]. It has been shown that oxidative stress imposed by ozone decreases lung glutathione levels associated with altered ECM protein type I collagen gene expression [229]. Other forms of oxidative stress which are derived from *t*-butyl hydroperoxide and iron could also modify collagen synthesis, by a mechanism presumably involving a redox sensor/receptor [226, 230]. TYAGI and coworkers [11, 226] demonstrated that *in vitro* exposure of normal myocardium to GSSG leads to the activation of MMPs, which was associated with oxidation of the active cysteine residue present on MMPs. GSSG also inhibited tissue inhibitor of metalloproteinase (TIMP) in fibroblasts [226]. Thiol antioxidants such as GSH and NAC and the phenolic antioxidant PDTC have been shown to inhibit the activation of MMP-2 and MMP-1 associated with increased TIMP levels in cultured fibroblast cells [220].

Immune effector response

The recent findings of PETERSON *et al.* [231] regarding the importance of GSH levels in antigen-presenting cells in modulating T-helper (Th) cell 1 *versus* Th2 cytokine response patterns in immune responses to the nature of the antigen, and the decreased intracellular levels of GSH in peripheral blood lymphocytes of CF and HIV-seropositive patients [144, 232], led to the assumption that this important tripeptide thiol may be involved in the

functional regulation of the immune response. However, the impact of chronic GSH depletion in T-cells, B-cells, macrophages and neutrophils in immune/inflammatory lung diseases have not been studied so far. Chronic depletion of GSH may be coupled with immunodeficiency and poor survival, as evidenced in the CD4 T-cells of HIV-seropositive patients [8, 21, 232, 233]. Intracellular redox GSH levels in these immune/inflammatory cells may also effect signal transduction and activation of transcription factors and lead to elevated gene expression (e.g. of IL-8 and IL-4) [8, 234]. GSH deficiency also leads to T-cell inactivation and apoptosis [235, 236]. More research is necessary to understand the mechanism/involvement of redox GSH levels and the regulation of glutathione synthesis in the regulation of the immune response.

Protective role of thiol compounds in inflammation

NAC, a cysteine-donating compound, acts as a cellular precursor of GSH and on deacetylation becomes cysteine. It reduces disulphide 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) [237]. NAC has been used in an attempt to enhance lung GSH levels and reduce inflammation in patients with COPD and IPF with variable success [238–241]. Oral treatment with NAC (200 mg three times daily for 8 weeks) in healthy chronic cigarette smokers reduced inflammation and lowered BALF levels of eosinophilic cationic protein, lactoferrin, antichymotrypsin and chemotactic activity for neutrophils [242].

In some cases, NAC might cause elevation of the antioxidant screen in lung ELF, as shown in patients with IPF, in whom NAC caused significantly elevated GSH levels in alveolar lavage fluid [241]. This may provide therapeutic effects on the rate and extent of the development of fibrotic lesions in these patients. Indeed, oral administration of 600 mg NAC three times daily for 12 weeks to patients with IPF improved lung function in these patients [243]. Intravenous NAC treatment for 72 h improved systemic oxygenation and reduced the need for ventilatory support in patients with mild-to-moderate acute lung injury but failed to influence the development of the condition, or its mortality [244]. In a prospective randomized double-blind placebo-controlled study, administration of NAC (150 mg·kg⁻¹ bolus, followed by a continuous infusion of 50 mg·kg⁻¹ over 4 h) led to decreased IL-8 levels without any change in plasma TNF- α , IL-6 or IL-10 levels in patients with septic shock [245]. This anti-inflammatory effect of NAC was associated with improved oxygenation and static lung compliance in these patients, suggesting that NAC may be used as an adjunctive treatment in patients with septic shock [245]. In an *in vitro* study, NAC was shown to inhibit neutrophil and monocyte chemotaxis and the respiratory burst [246]. Animal studies have suggested that NAC produces deleterious effects on the lung epithelium in response to hyperoxia exposure [247]. Furthermore, a direct link between these clinical effects (*i.e.* reductions in the number of exacerbations and in the decline in lung function and inflammation) and the efficacy of NAC as an

in vivo antioxidant has not been convincingly established to date [1, 13].

NAL, a lysine salt of *N*-acetyl-L-cysteine, is a mucolytic and antioxidant thiol compound. The advantage of NAL over NAC is that it has a neutral pH in solution, whereas NAC is acidic. NAL can be aerosolized into the lung without causing significant side-effects [248]. GILLISSEN *et al.* [248] compared the effects of NAL and NAC and found that both drugs enhanced intracellular glutathione levels in alveolar epithelial cells and inhibited H₂O₂ and O₂^{·-} release from human blood-derived polymorphonuclear neutrophils (PMNs) from smokers with COPD. NAL also inhibited ROS generation induced by serum-opsonized zymosan by human PMNs. This inhibitory response was comparable to the effects of NAC [249]. Therefore, NAL may represent an interesting alternative approach to augmenting the antioxidant screen in the lungs.

Certain other thiol-releasing agents such as glutathione ethyl ester and L-thiozolidine-4-carboxylate are potentially useful compounds for cysteine/glutathione delivery [250, 251]. However, studies are needed to validate the bio-availability of these compounds in lung inflammation.

Glutathione therapeutic perspectives in inflammatory lung diseases

The evidence is overwhelming that glutathione plays a vital role in cellular modulation of the inflammatory response, antioxidant capability, antiprotease system, immune effectiveness, remodelling of the ECM, surfactant/phosphatidylcholine and mucolysis. It is clear that ELF GSH levels are decreased in various inflammatory lung diseases. Thus increasing lung cellular levels of the GSH/antioxidant screen would be a logical approach in inflammatory lung diseases. Extracellular augmentation of GSH has been attempted through intravenous administration of GSH, oral ingestion of GSH and inhalation of nebulized GSH [252–260] in an attempt to reduce inflammation in lung diseases [261] such as in IPF [253], mild asthmatics [254] and CF [255, 256]. GSH aerosol therapy normalized low GSH levels in the lungs of these patients [253–256]; however, nebulized GSH also had a detrimental effect in asthmatic patients by producing bronchoconstriction, presumably due to the formation of GSSG [254]. This suggests that GSH aerosol therapy may not be an appropriate means of increasing GSH levels in lung ELF and cells in patients with asthma. GSH aerosols also increased the formation of GSSG in patients with IPF [253] but suppressed lung epithelial surface inflammatory cell-derived oxidants in patients with CF. Recent data have indicated that the low levels of ELF/apical fluid GSH in patients with CF is attributable to abnormal GSH transport (inability to bring out GSH efflux), which might be associated with a missing or defective CF transmembrane conductance regulator channel, through which efflux of organic anions normally occurs [262, 263]. The depletion of lung GSH may be reflected in peripheral blood lymphocytes in these patients [144]. It may be feasible to use a GSH aerosol to restore the oxidant/antioxidant imbalance in these patients [256, 259]. In all of these studies, questions were raised as to the bio-availability of GSH, the pH and osmolality at the site of

the microenvironment and the resultant formation of deleterious products (GSSG). It seems rational to suggest that neutralizing the pH, providing GSH in salt form, using liposome-entrapped GSH delivery and maintenance of isotonicity would be useful in designing any GSH inhalation therapy in inflammatory lung diseases.

Increasing the activity of γ -GCS and glutathione synthetase by gene transfer techniques may increase cellular GSH levels. Transfection of cDNAs for the heavy and light subunits of human γ -GCS-HS resulted in elevation of intracellular glutathione levels in COS-7 cells [264]. These cells were thereafter resistant to chemotherapeutic drugs. Similarly, MANNA *et al.* [265] have recently demonstrated that overexpression of γ -GCS in rat hepatoma cells completely protected against the TNF- α -induced activation of NF- κ B, AP-1, stress-activated protein kinase/JNK and apoptosis. They highlighted the importance of glutathione and γ -GCS in protecting against the cytotoxic effects of various agents and that most of the actions of TNF- α are regulated by the glutathione-controlled redox status of the cell. Modulation of GSH synthesis in organs such as the skeletal muscle of critically ill patients, in whom GSH synthesis is markedly affected, would be another means of targeting GSH synthesis by gene transfer [259, 266]. Thus the induction of γ -GCS by molecular means to increase cellular GSH levels or γ -GCS gene therapy holds great promise in protection against chronic inflammation and oxidant-mediated injury in various inflammatory diseases.

Conclusions and future directions

ROS and RNS are generated by several inflammatory and immune and various structural cells of the airways. An imbalance of oxidant/antioxidant in favour of oxidants contributes to the pathogenesis of several inflammatory lung diseases. GSH is an important protective antioxidant in the lungs, the levels of which are altered in the ELF in several of these conditions. Glutathione and γ -GCS are regulated by oxidants, phenolic antioxidants, pro-inflammatory mediators and anti-inflammatory agents and growth factors in lung cells. In addition, intracellular and extracellular GSH levels are also regulated by cystine/cysteine transport mechanisms and by γ -GT. γ -GCS is regulated at both transcriptional (AP-1, ARE and NF- κ B) and translational levels. Although the molecular mechanism of γ -GCS activity is well characterized, its regulation in response to specific stimuli in particular inflammatory lung diseases has not been studied. More work should be carried out to address this regulation using human tissue.

Regulation of intracellular redox GSH levels in response to ROS/RNS and in inflammation should have critical effects, in different lung cells, on the activation of redox sensor/signal transduction pathways and various transcription factors such as NF- κ B and AP-1. The regulation of intracellular glutathione levels may determine the balance between expression of pro-inflammatory mediators and antioxidant genes. Imbalance of redox-regulated pro-inflammatory and antioxidant genes in response to inflammatory mediators, oxidants and growth factors might be associated with susceptibility/tolerance to disease. Modern technologies such as DNA array and differential display might be utilized in detecting and assessing the novel

target genes involved in disease susceptibility. The polymorphism in the γ -GCS gene may be associated with initiation/exacerbation of inflammation as a result of a mutation in this critical antioxidant gene. Hence, studies must be directed at detecting the existence, if any, of such genetic polymorphisms in susceptible/nonsusceptible populations and patients with inflammatory lung diseases.

Study of the protective role of reduced glutathione/thiol compounds in inhibition of the inflammatory response (activation of nuclear factor- κ B/inhibitor of nuclear factor- κ B kinases) and correcting the fundamental oxidant/antioxidant imbalance in patients with chronic inflammatory diseases are important areas of further research. Augmentation of intra- and extracellular levels of glutathione and related thiols *via* aerosolization/inhalation and γ -glutamylcysteine synthetase by gene transfer in lungs will not only enhance the protective antioxidant potential but may also inhibit oxidant-mediated acute and chronic inflammatory responses. Understanding of the cellular and molecular redox-regulating mechanisms in inflammation is needed to design antioxidant therapeutic strategies for the treatment of various inflammatory lung conditions.

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