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Citation for published version:

Biswas, SK & Rahman, I 2009, 'Environmental toxicity, redox signaling and lung inflammation: the role of glutathione' *Molecular aspects of medicine*, vol 30, no. 1-2, pp. 60-76., 10.1016/j.mam.2008.07.001

Digital Object Identifier (DOI):

[10.1016/j.mam.2008.07.001](https://doi.org/10.1016/j.mam.2008.07.001)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Author final version (often known as postprint)

Published In:

Molecular aspects of medicine

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Published in final edited form as:

Mol Aspects Med. 2009 ; 30(1-2): 60–76. doi:10.1016/j.mam.2008.07.001.

ENVIRONMENTAL TOXICITY, REDOX SIGNALING AND LUNG INFLAMMATION: THE ROLE OF GLUTATHIONE

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Abstract

Glutathione (γ -glutamyl-cysteinyl-glycine, GSH) is the most abundant intracellular antioxidant thiol and is central to redox defense during oxidative stress. GSH metabolism is tightly regulated and has been implicated in redox signaling and also in protection against environmental oxidant-mediated injury. Changes in the ratio of the reduced and disulfide form (GSH/GSSG) can affect signaling pathways that participate in a broad array of physiological responses from cell proliferation, autophagy and apoptosis to gene expression that involve H_2O_2 as a second messenger. Oxidative stress due to oxidant/antioxidant imbalance and also due to environmental oxidants is an important component during inflammation and respiratory diseases such as chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, acute respiratory distress syndrome, and asthma. It is known to activate multiple stress kinase pathways and redox sensitive transcription factors such as Nrf2, NF- κ B and AP-1, which differentially regulate the genes for pro-inflammatory cytokines as well as the protective antioxidant genes. Understanding the regulatory mechanisms for the induction of antioxidants, such as GSH, versus pro-inflammatory mediators at sites of oxidant-directed injuries may allow for the development of novel therapies which will allow pharmacological manipulation GSH synthesis during inflammation and oxidative injury. This article features the current knowledge about the role of GSH in redox signaling, GSH biosynthesis and particularly the regulation of transcription factor Nrf2 by GSH and downstream signaling during oxidative stress and inflammation in various pulmonary diseases. We also discussed the current therapeutic clinical trials using GSH and other thiol compounds, such as N-acetyl-L-cysteine, fudosteine, carbocysteine, erdosteine in environment-induced airways disease.

Keywords

Reactive Oxygen Species; Glutathione; Nrf2; inflammation; lung; COPD

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1. Introduction

1.1. Oxidative stress and lung toxicity

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play important roles in regulation of cell survival. Normally, moderate levels of ROS/RNS may function as signals to promote cell proliferation and survival, whereas a sudden and prolonged surge of ROS/RNS can induce cell death. Under normal physiologic conditions, the balance between generation and elimination of ROS/RNS maintains the functional integrity of redox-sensitive signaling proteins. The redox homeostasis of a cell ensures that endogenous and exogenous stimuli are modulated by the redox homeostasis of a cell. However, altered redox homeostasis leads to oxidative stress, which in turn may lead to aberrant cell death and contribute to disease development (Trachootham et al., 2008).

The lung, owing to its extensive surface area and blood supply, is the only organ in the entire human architecture which has the greatest exposure to atmospheric oxygen and other environmental toxicants. Hence, the lung is susceptible to oxidative injury by virtue of myriads of reactive forms of oxygen species. ROS and RNS are highly unstable due to unpaired electrons that are capable of initiating oxidation (reviewed in Valko et al., 2007). *In situ* lung injury due to ROS/RNS is linked to oxidation of proteins, DNA, and lipids. Physiologically, ROS/RNS inflict their effects by remodeling the extracellular matrix and blood vessels; stimulate mucus secretion and alveolar repair responses. At the biochemical level, ROS/RNS inactivate antiproteases, induce apoptosis, regulate cell proliferation and modulate the immune-inflammatory system in the lungs and other tissues (Rutkowski et al., 2007). At the molecular level, increased ROS/RNS levels have been implicated in initiating inflammatory responses in the lungs through the activation of transcription factors such as nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1), signal transduction, chromatin remodeling and gene expression of pro-inflammatory mediators (Rutkowski et al., 2007). This article will discuss the current knowledge about the role of glutathione (GSH) in redox signaling, GSH biosynthesis and particularly the regulation of transcription factor, nuclear redox factor2 (Nrf2) by GSH and downstream signaling during oxidative stress and inflammation in various pulmonary diseases. It will also discuss the current therapeutic clinical trials using GSH and other thiol compounds, such as N-acetyl-L-cysteine (NAC), fudosteine, carbocysteine, erdosteine in airways disease.

2. Oxidant sources in the lungs

2.1. Cell-derived endogenous ROS/Oxidants

Lung is vulnerable to oxidant damage because of its location, anatomy and function (Crystal, 1991). Lung epithelium is constantly exposed to oxidants generated internally as a part of normal metabolism, as well as to oxidants in the ambient air, including ozone, nitrogen dioxide, car exhaust, cigarette smoke etc. A free radical is any species capable of independent existence that contains one or more unpaired electrons (Halliwell and Gutteridge, 1999). The most important ROS of physiological significance are the superoxide anion ($O_2^{\bullet-}$), hydroxyl radical (OH^{\bullet}), nitric oxide (NO), and hydrogen peroxide (H_2O_2). H_2O_2 is generated through nonenzymatic or enzymatic dismutation of superoxide. However, the most reactive and harmful ROS is OH^{\bullet} , which can be formed from H_2O_2 and superoxide but also via the reaction of superoxide with NO to produce peroxynitrite ($ONOO^-$), which decomposes to form nitrogen dioxide (NO_2) and OH^{\bullet} . The main cellular sources of ROS in the lung include not only neutrophils, eosinophils and alveolar macrophages (Kinnula et al., 1995), but also alveolar epithelial cells, bronchial epithelial cells and endothelial cells (Kinnula et al., 1992a, 1992b).

2.2. Inhaled Oxidants

The generation of ROS in the lungs is enhanced after exposure to numerous exogenous environmental chemical and physical agents, which include mineral dusts, ozone, nitrogen oxides, sulphur dioxide, ultraviolet and ionizing radiation (Janssen et al., 1993), and tobacco smoke (Church and Pryor, 1985). A direct consequence of cigarette smoking and inhalation of airborne pollutants is lung damage and elevated inflammatory responses in the lungs, and are implicated in the pathogenesis and exacerbations of chronic obstructive pulmonary disease (COPD). In the gas phase, the cigarette smoke comprises of high concentrations of oxidants/free radicals ($>10^{15}$ molecules/puff) (Church and Pryor, 1985), short-lived oxidants such as $O_2^{\cdot-}$ and NO. Nitric oxide and $O_2^{\cdot-}$ immediately react to form highly reactive ONOO⁻ molecule. The tar phase of cigarette smoke contains organic radicals, such as long-lived semiquinone radicals, which can react with molecular oxygen in a redox dependent manner to form $O_2^{\cdot-}$ to form OH[•] and H₂O₂ (Nakayama et al., 1989). The aqueous phase of cigarette smoke condensate may undergo redox recycling for a considerable period of time in the epithelial lining fluid (ELF) of smokers (Nakayama et al., 1989). The tar phase is also an effective metal chelator wherein iron is chelated to produce tar-semiquinone + tar-Fe²⁺, which can generate H₂O₂ continuously (Nakayama et al., 1989). It is of much importance to note that cigarette smoke has been recently reported to irreversibly modify GSH levels in airway epithelial cells; a basic mechanism which may underlie cigarette smoke mediated inflammatory lung damage (van der Toorn et al., 2007).

3. GSH Biosynthesis and Regulation

GSH is the predominant non-protein thiol in the cells and is a key player in the maintenance of the cellular redox status, defined as the ratio of the concentration of oxidizing equivalents to that of reducing equivalents (Forman and Dickinson, 2003). GSH exists primarily in two redox forms, i.e., reduced GSH and glutathione disulfide (GSSG, the oxidized form), the latter representing a negligible 1/100th of the total GSH pool. The normal GSH content of a cell ranges from 1 mM-10 mM, levels that are imperative for a cell to maintain and a function of the balance between depletion and synthesis. Cells can excrete GSSG or reduce it back to GSH at the expense of NADPH through the action of GSH reductase. However, *de novo* synthesis of GSH from its amino-acid constituents is essential for the elevation of GSH that occurs as an adaptive response to oxidative stress. GSH synthesis involves two enzymatic steps catalyzed by glutamate cysteine ligase (GCL, formerly called as γ -glutamylcysteine synthetase) and GSH synthetase (Meister and Anderson, 1983). The enzyme GCL controls the rate of GSH synthesis (Huang et al., 1993) because the rate-limiting step for *de novo* synthesis of GSH is the cellular levels of the amino acid cysteine. In that regard, the plasma membrane ectoenzyme γ -glutamyl transpeptidase (γ -GT), which is the only enzyme that can break the γ -linkage found in GSH and GSH-conjugates, is essential in providing cysteine. It metabolizes the extracellular GSH and preferentially forms γ -glutamylcysteine, which is taken up by cells and subsequently reduced to form γ -glutamylcysteine bypassing its production by GCL.

GCL is composed of a heterodimer containing a 73 kDa heavy catalytic subunit (GCLC) and a 30 kDa light modifying subunit (GCLM) (Huang et al., 1993). Although the heavy subunit contains all of the catalytic activity, the association of the heavy subunit with the regulatory light subunit can modulate GCL activity. The ratio of the two subunits for physiological function has long been assumed to be 1:1; however, in tissues the ratio varies significantly and usually GCLC:GCLM is significantly greater than 1:1 (Krzywanski et al., 2004). GCL is regulated by GSH through feedback inhibition. Changes in GCL activity can result from regulation at multiple levels affecting only the catalytic or modifier subunit or both (Lu, 1999). Both human GCLC and GCLM promoters have been cloned (Erickson et al., 2002; Galloway et al., 1997). GCL has been shown to be polymorphic and is directly associated with

loss in lung function and smoking history (Siedlinski et al., 2008). However, Chappell et al., have found that polymorphism of GCL is not associated with COPD (Chappell et al., 2008). Very recently, it has been reported that patients with cystic fibrosis (CF) with a milder *CFTR* genotype, exhibit a strong association between functional polymorphisms of the *GCLC* gene and CF severity (McKone et al., 2006). This study was mainly prompted by an earlier observation that patients with CF have low levels of GSH in the epithelial lining fluid (ELF) (Roum et al., 1993).

3.1 GSH: Oxidative stress and Redox Regulation in Lung Cells

The presence of sulfhydryl group in GSH makes it function as an antioxidant, protecting against free radicals and other oxidants. Increasing intracellular GSH can decrease the release of cytokines and chemokines from lung cells by decreasing NF- κ B activation (Antonicelli et al., 2002; Aoki et al., 1996). In addition, GSH is an important regulator of cell proliferation, apoptosis, and gene transcription (Rahman and MacNee 2000a; Luppi et al., 2005) and can be easily assayed in the laboratory (Rahman et al., 2006). Reduced GSH is distributed relatively evenly throughout the cell, with the exception of the lumen of the rough endoplasmic reticulum (ER), where very little is detected. Both endogenous as well as exogenous oxidants require hours to significantly affect GSH levels in the majority of cells in a given population (Ault and Lawrence 2003). Interestingly, cells within a homogeneous cell line population lose GSH at different rates. The last reserves of cellular GSH are however, found within the mitochondria (Ault and Lawrence 2003). GSH and GSSG form one of the most important redox couple in a cell. GSH is not restricted to a particular cell organelle, rather there is a dynamic distribution going on depending on the cell's redox status (Beck et al., 2001). GSH pathways/bioactivities may greatly differ from cell compartment to cell compartment (mitochondria, ER, cytoplasm and nucleus) and cell to cell (cancer cell, epithelial cells, immune system cells) (Table 1), and airway surface liquid compartments (airway surfaces, alveolar surfaces) and hence the cytosolic redox ratios of GSH/GSSG will be a reflective of any of these specific sub-compartments.

GSH is vital in the lung, defending the airspace epithelium from damage in response to oxidants and inflammation and GSH redox status is critical for the transcriptional regulation of many pro-inflammatory genes (Figure 1). This is illustrated by findings in various pulmonary diseases where decreases in the levels of GSH in the lung lining fluid have been shown to occur in idiopathic pulmonary fibrosis (IPF), acute respiratory distress syndrome (ARDS), CF, lung allograft patients and human immunodeficiency virus (HIV) positive patients (Rahman and MacNee, 1999 and 2000a) (Table 2). In contrast, an increase in total GSH concentration, which also includes the oxidized form GSSG, has been reported in the bronchial and alveolar fluid in patients with mild asthma (Rahman and MacNee, 2000b). Furthermore, GSH has recently been reported to attenuate IL-13 induced asthma in mice (Lowry et al., 2008). Thus, low levels of GSH in the lung lining fluid of patients with inflammatory respiratory diseases may render them more susceptible to the deleterious effects of subsequent exposure to inhaled toxicants and may also perpetuate the inflammatory response. It has now become evident that small changes in the cellular redox status may alter signaling pathways.

The GSSG/GSH ratio can serve as a good indicator of the cellular redox state (Park et al., 1998). A very recent report has suggested that changing GSH redox balance, increasing GSH level, and the GSH/GSSG ratio by γ -glutamylcysteinyl ester (γ -GCE), ameliorate bronchial asthma by altering the Th1/Th2 cell imbalance through IL-12 production from antigen presenting cell (APC) and suppressing chemokine production and eosinophil migration itself (Koike et al., 2007). Although the ER may be the major site of GSSG accumulation, the formation of GSSG from GSH due to oxidation in the cytosol triggers dynamic redistribution of GSH and GSSG between their respective organelles and the cytosol (Banhegyi et al.,

1999, Banhegyi et al., 2003). Hence, cytosolic GSH concentration is important for biological cellular redox state. It is noteworthy that high levels of GSSG do not necessarily reflect that ER is storage or accumulation point for GSSG but as per recent reports by itself is an important generator of oxidative stress. Therefore more GSH is oxidized to GSSG in the ER (Chakravarti et al., 2006). Besides, GSH is known to be an important redox buffer within the ER and is required to balance the ER generated oxidative stress (Jessop and Bulleid 2004).

Several enzymes/proteins involved in the redox system of the cell and their genes such as MnSOD, GCLC, GSH peroxidase (GPx), thioredoxin (Trx) reductase, glutaredoxin1 and metallothionein are induced by modulation of cellular GSH/GSSG levels in response to various oxidative stresses, including hyperoxia and inflammatory mediators such as tumor necrosis factor- α (TNF- α) and lipopolysaccharide in lung cells (Das, 2001; Peltoniemi et al., 2006). It has been shown that GSH depletion due to GCL inhibition by buthionine sulphoximine (BSO) sensitizes both alveolar A549 and bronchial epithelial 16-HBE cells to the injurious effects of hyperoxia and H₂O₂, resulting in an increased membrane permeability and activation of NF- κ B (Rahman et al., 2001). In contrast, pre-treatment of these cell lines with hyperoxia prior to H₂O₂ exposure protects against the cytotoxic effects of H₂O₂ as well as preventing NF- κ B activation. These protective effects were due to an adaptive increase in GSH in response to pre-treatment with hyperoxia. Therefore, modulation of intracellular GSH can determine the course of tolerance to subsequent oxidant exposure. A recent study has indicated that oxidant induced suppression of GSH synthesis in human bronchial cells may be reversed by erythromycin (He et al., 2008). However, more such studies are required to validate the results so as to have a therapeutic impact.

Normally, GSSG represents less than 1% of total GSH pool. When H₂O₂ or ONOO⁻ is transiently elevated, an elevation in GSSG, also transient, can occur, providing a possible mechanism for signaling by means of thiol-disulfide exchange. In this scenario, signaling is indirectly dependent upon ROS generation. Nonetheless, as this mechanism requires a change in GSSG that is usually only observed during oxidative stress, such signaling is more likely an oxidative stress response rather than physiologic redox signaling. Interesting to note is a recent report wherein it was shown that the sputum levels of GSSG and nitrosothiols such are elevated in COPD subjects the increase was associated with neutrophilic inflammation (Beeh et al., 2004). Therefore, increased GSSG is not only an indicator for oxidative stress but its presence in the sputum may serve as a marker of oxidative stress in lung diseases.

There have been recent interests in the role of thiol containing enzymes in redox regulation of signaling pathways which is comprehensively reviewed by Bindoli et al. (Bindoli et al., 2008). Peroxiredoxins are a class of enzymes that can reduce H₂O₂ using thiols (Rhee et al., 1999) in a reaction similar to that catalyzed by the selenium in GPx, some using Trx as a co-substrate and at least one using GSH (Chen et al., 2000). Interestingly, they contain reactive cysteine residues in their unprotonated form, i.e. thiolate (S⁻) and the catalytic reaction includes several steps, one of which being the formation of a protein-sulfenate intermediate (PSO⁻), which then react with a thiol that can be the second reactive thiol present in most 2-Cys peroxiredoxins (Wood et al., 2003a) or GSH (Chen et al., 2000). Peroxiredoxins might play an important role in controlling redox signaling pathways (Wood et al., 2003b). Transgenic mice overexpressing peroxiredoxin6 have been demonstrated to show increased resistance to lung injury during hyperoxia (Wang et al., 2006). The levels of another thiol, glutaredoxin, have been shown to be modulated in the lungs and sputum of smokers and in COPD subjects and in mouse models of allergic airway diseases (Peltoniemi et al., 2006; Reynaert et al., 2007). The role of various thiols and the mechanism of their redox modulation leading to parenchymal lung diseases have been reviewed in details by Kinnula et al. (Kinnula et al., 2007). Protein disulfide bonds rarely form in the cytosol because of the high concentrations of GSH. By contrast, a relatively higher concentration of GSSG exists in the ER lumen (Hwang

et al., 1992). Such an oxidizing condition within the ER lumen allows the formation of native disulfide bonds in the ER through a complex process involving not only disulfide-bond formation, but also the isomerization of non-native disulfide bonds, i.e. folding of proteins (Chakravarti et al., 2006). Very recently Bindoli et al. have suggested that the status of thiol redox may be attributed to the oxidant status of the cell (Bindoli et al., 2008). The thiol redox state is modulated by Trx and GSH systems that are in close redox link with H₂O₂ via peroxiredoxins and GPx, respectively. Particularly, the redox state of Trx integrates the extent of cell oxidation to a rather specific physiologic response, which may be different in different cellular compartment. For example, in the mitochondria the redox state of Trx influences the membrane-permeability conditions, in the cytosol controls the mitogen activated protein kinase (MAPK) signaling pathways, and within the nucleus regulates the binding of transcription factors to DNA.

4. Regulation of GSH biosynthesis by pro-inflammatory mediators and growth factors

The synthesis of GSH is upregulated during oxidative stress and inflammation. Thus, an increase in GCL expression would be expected under oxidative stress and the first demonstration of that was with the redox cycling and GSH conjugating quinone and menadione (Shi et al., 1994). Exposure of alveolar epithelial cells *in vitro* to oxidants, oxidant-generating systems, and lipid peroxidation products, such as H₂O₂, hyperoxia, ozone, menadione, and 4-hydroxy-2-nonenal, all lead to short-term falls in intracellular GSH associated with increased GSSG levels, followed by increases in GSH levels or upregulation of GCLC mRNA in alveolar epithelial cells, endothelial and other cells *in vitro*, and *in vivo* in rats (Paget et al., 1998; Parmentier et al., 2000; Pietarinen-Runtti et al., 1998; Usatyuk et al., 2006). A recent report indicates that chronic alcoholism may also lead to alteration of systemic and pulmonary GSH redox system (Yeh et al., 2007). The GCLM is also concomitantly induced in response to oxidants and phenolic antioxidants in rat lung epithelial L2 cells, suggesting that concomitant induction of both subunits may be a potential mechanism to enhance cellular GSH synthesis, and so develop cellular tolerance to oxidative stress (Tian et al., 1997). While the mRNAs for both subunits increase in response to oxidative stress, there may be a disproportional increase in the proteins (Krzywanski et al., 2004). Thus, the short-term effects of various oxidants and oxidant-generating systems appear to upregulate the gene for GSH synthesis, possibly providing a protective/adaptive mechanism against subsequent oxidative stress.

Identification and characterization of the types of diverse stimuli that act as potent inducers of GCL should aid in the development of effective pharmacological strategies for antioxidant treatment involving GSH regulation in inflammatory lung diseases. To this effect, 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. Several investigators have reported that the promoter (5'-flanking) region of human GCLC gene is regulated by a putative c-Jun homodimeric complex-AP-1 sequence (Tanaka et al., 1998; Tomonari et al., 1997). This sequence is located at the proximal region of the GCLC TATA box in various cell lines, including human alveolar epithelial cells (Tanaka et al., 1998; Tomonari et al., 1997). Mulcahy and co-workers (Monva and Mulcahy, 1998; Mulcahy et al., 1997), however, have reported a distal antioxidant response element (ARE) containing an embedded phorbol myristate acetate (PMA)-responsive element (TRE/AP-1) and an electrophile responsive element (EpRE or its functional equivalent, ARE), which play a key role in the regulation of the GCLC and GCLM, respectively, in response to a planar aromatic xenobiotic, the phenolic antioxidant β -naphthoflavone specifically in a liver cell line (HepG2 cells) (Erickson et al., 2002). They further suggested a differential induction of mafF, mafG and mafK expression by electrophile-

response-element activator in the regulation of GCLC regulation in a variety of cell lines (Moran et al., 2002). It has been shown that H₂O₂-dependent activation of GCLC-ARE4 reporter occurs via the MAPK pathways without oxidation of cellular GSH or Trx-1 suggesting that redox GSH status of the cells is not required for regulation of GCLC or ARE (Harper et al., 2001). Mulcahy and colleagues also showed that the internal AP-1 site is important for the constitutive expression of the *GCLM* gene (Monva and Mulcahy 1998). However, Galloway and co-workers (Galloway et al., 1997) were unable to demonstrate a role for ARE in the induction of GCLM by oxidants such as *tert*-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 GCL 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)-*tert*-butylated-4-hydroxyanisole and butylated hydroxytoluene as well as the synthetic indolic antioxidant, 5,10-dihydroindeno(1,2-b) indole and pyrrolidine dithiocarbamate, a sulfhydryl-modifying antioxidant compound, upregulate GCLC and GCLM in human endothelial cells and other cell lines (Galloway et al., 1997; Tu and Anders, 1998). These effects of phenolic antioxidants are associated with AP-1/ARE transactivation (Meyer et al., 1993; Pinkus et al., 1996). Taken together, antioxidants protect cells from oxidants by either scavenging these molecules directly or by regulating intracellular GSH levels through the induction of GCL. It should however, be kept in mind that recent studies have shown that many compounds characterized originally as antioxidants have been also shown to exert direct effects upon several signal transduction enzymes independent of their antioxidant function (Dickinson et al., 2003; Biswas et al., 2005; Kode et al., 2008). For example vitamin E has significant effects upon signaling independent of its lipid peroxidation inhibitory role (Azzi et al., 1998).

Transforming growth factor- β 1 (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 cells. De Boer have shown increased expression of TGF- β 1 in bronchiolar and alveolar epithelium in idiopathic IPF and COPD patients, and higher levels in BAL of atopic asthmatics as compared to healthy subjects (de Boer et al., 1998). TGF- β 1 also down-regulates GCLC mRNA and GSH synthesis in *in vitro* human alveolar epithelial cells and pulmonary artery endothelial cells (White et al., 1992). We, and other workers have shown that GCLC mRNA expression is under the control of the AP-1-like transcription factor (Rahman et al., 1996a), and that TGF- β 1 may decrease GCLC gene expression via an ATF4/Nrf2/ARE-1-dependent mechanism (Jardine et al., 2002; Bakin et al., 2005). It has been shown that downregulation of GCLC in response to TGF- β 1 in lung epithelial cells was mediated via an AP-1 heterodimer consisting of c-Jun and Fra-1 (Jardine et al., 2002) whereas another study has reported that ATF3, a negative regulator of Nrf2-ARE binding, upregulated by TGF- β 1 downregulated Nrf2 by sequestering p300 protein away from Nrf2 (Bakin et al., 2005). Thus, higher levels of TGF- β 1 may down-regulate GSH synthesis in lungs of patients with inflammatory diseases such as IPF and COPD. It has been shown that GSH levels in bronchoalveolar lavage fluid (BALF) is decreased during exacerbations of COPD though GSH levels were increased in BALF of smokers and stable COPD patients compared to non-smokers (Drost et al., 2005). Recent studies have clearly highlighted the downregulation of Nrf2 (GSH levels) in pulmonary macrophages and in lungs of patients with COPD via loss of GCL and Nrf2 positive regulator DJ-1 and post-translational modifications of Keap1-Bach1 equilibrium (Harju et al., 2002; Malhotra et al., 2008; Goven et al., 2008; Suzuki et al., 2008). Moreover, decreased GSH-levels may also have direct functional consequences leading to inflammation. *In vitro* studies showed that GSH (in the concentration range normally found in ELF) suppressed fibroblast proliferation (Cantin et al., 1990). In addition, depletion of GSH in response to TGF- β 1 appears to be a key requirement for subsequent collagen I mRNA expression in murine embryo fibroblasts (Liu et al., 2004). This

induction was attenuated by pre-treatment with NAC, GSH, or GSH ester. The relevance of GSH regulation and subsequent tolerance/susceptibility in lung epithelial cells in response to pro-/anti-inflammatory mediators and/or oxidants under chronic inflammation *in vivo* is presently not clearly understood.

4.1 Redox regulation of ARE (Nrf and Maf proteins)

Transcription factor Nrf2 is an essential component for the ARE-mediated induction of phase II detoxifying and GCL genes in response to electrophiles and phenolic antioxidants in HepG2 cells (Wild et al., 1999). It is now known that Nrf proteins bind to the ARE consensus sequence, which shows striking similarity to a binding motif referred to as the Maf recognition element, also known as the erythroid transcription factor (NF-E2) binding sequence. NF-E2 or Maf recognition elements are specifically recognized by either homodimers of small Maf family members (MafK, MafG, MafF) or by heterodimeric proteins composed of Cap'n'Collar (CNC) subfamily of basic leucine zipper (bZip) transcription factors and small Maf partners which mediate expression through AP-1/EpRE sequences. The NF-E2/AP-1 element and the ARE, which has been identified in several phase II genes, possesses striking similarity.

A protein Kelch-like ECH-associated protein1 (Keap1) which plays a role in the ARE-mediated signaling, has been identified as a negative regulator of Nrf2 that also acts as a possible intracellular sensor for thiol-active xenobiotics in HepG2 cells (Itoh et al., 1999). Keap1 binds and retains Nrf2 in an inactive form in the cytoplasm, a form in which Nrf2 is unable to function as a transcription factor. During transition to oxidizing conditions (represented by oxidation of GSH by ROS to GSSG), Nrf2 dissociates from Keap1 allowing it to translocate to the nucleus where it heterodimerizes with small Maf proteins and activates ARE-driven transcription (Dinkova-Kostova et al., 2002). The actual mechanism of dissociation of Nrf2 from Keap1 is currently not clear, but it is thought to involve thiol modifications or phosphorylation of Keap1, Nrf2, or both or via kinase signaling mechanism. Indeed, there is evidence in a cell-free system that cysteine thiols of Keap1 are reactive to inducers of phase 2 genes (Dinkova-Kostova et al., 2002). Four of the most reactive cysteines resided in the region required for binding to Nrf2. Exposure of the Keap1/Nrf2 complex to the inducers resulted in the dissociation of the complex. Thus, the sulfhydryl groups of Keap1 are sensors regulating the induction of phase 2 enzymes (Wakabayashi et al., 2004). Therefore, under the conditions of initial depletion of intracellular GSH, Trx and/or production of ROS may lead to the dissociation of the complex and activation of Nrf2 (Kim et al., 2003). Furthermore, it has been shown that scaffolding of Keap1 to the actin cytoskeleton controls the function of Nrf2, and under oxidative stress and electrophilic stress disruption of the actin cytoskeleton promotes nuclear entry of an Nrf2 reporter protein (Kang et al., 2004).

It has been also demonstrated that mice with site-directed mutation of the Nrf2 gene (Nrf2^{-/-}) were more susceptible to the injurious effects of hyperoxia as noted by marked increase in pulmonary hyper permeability, macrophage inflammation, and epithelial injury compared to the wild type mice (Hayes and McLellan, 1999). A significant reduction in the mRNA expression of ARE-responsive lung antioxidant and phase 2 enzymes, some of which included heme-oxygenase-1, GPx2, and NAD(P)H: quinone oxidoreductase, was also observed in Nrf2^{-/-} mice compared to normal mice. GPx2, the major cigarette smoke-inducible isoform has now been confirmed to be regulated by Nrf2 (Singh et al., 2006). Thus Nrf2 appears to protect against pulmonary hyperoxia injury in mice, presumably by upregulating the transcription of lung antioxidant defense enzymes. However, it remains to be explained as to the role of Keap1 and Nrf2, if any, in the induction of phase II genes in particular GCL in lung cells in response to oxidative stress. Keap1 also appears to regulate the degradation of Nrf2 via a proteasome-dependent pathway and that mouse macrophages deficient in Keap1, Nrf2 accumulates in the nucleus (Zhang and Hannink, 2003). Electrophilic compounds have also been shown to cause

Nrf2 nuclear translocation, concomitant with protein stabilization. Furthermore, electrophilic lipids, like prostaglandins, isoprostanes and 4-hydroxy-2-nonenal also cause the dissociation of Nrf2 from Keap1, resulting in the activation of the ARE.

As described previously, signals from ROS or electrophilic insults target the Nrf2-Keap1 complex, dissociating Nrf2 from Keap1, leading to translocation of Nrf2 to the nuclei and transactivate the target genes. Keap1 is now assumed to be a substrate-specific adaptor of Cul3-based E3 ubiquitin ligase (Kobayashi and Yamamoto, 2005). Direct participation of Keap1 in the ubiquitination and degradation of Nrf2 is now thought to be plausible. The Nrf2-Keap1 system is present not only in mammals but also in fish suggesting that its roles in cellular defense are conserved throughout evolution among vertebrates (Kobayashi and Yamamoto, 2005). As discussed in the following section, Nrf2 has an important role in regulation of the GCLC and GCLM genes. Recently, it has been shown that GSH levels also determine the expression of Nrf2. In an elegant experiment by Lee et al. (Lee et al., 2008), it was shown that murine embryonic fibroblasts (MEFs) can survive in the presence of GSH inhibitor buthionine-(S,R)-sulfoximine (BSO), even though most intracellular GSH was depleted. As a cellular adaptive mechanism, BSO treatment effectively activated the Nrf2 pathway, which led to up-regulation of antioxidant enzymes in these cells through the extracellular signal-regulated kinase cascade. Nrf2-deficient MEFs lost the inducibility of antioxidant genes, which resulted in higher levels of ROS accumulation, caspase-3 activation, and cell death when compared to wild-type cells. On the other hand, Nrf2-deficient cells could be more sensitized to doxorubicin-induced cell death by BSO pre-incubation, while wild-type cells were not. In addition, BSO-mediated cell death was facilitated by administering Nrf2 siRNA to chemoresistant human ovarian cancer cells. These results indicated that Nrf2 is the primary factor inducing the cell survival system under GSH depletion.

The aryl hydrocarbon receptor (AHR) and NF-E2 p45-related factor (Nrf2) are two distinct transcription factors involved in the regulation of drug-metabolizing enzymes. Spate of evidences from several studies implies that AHR and Nrf2 have direct links, but the underlying molecular mechanism still remains to be fully explored. It was recently reported that Nrf2 gene transcription is directly modulated by AHR activation (Miao et al., 2005). DNA sequence analyzes of the mouse Nrf2 promoter revealed one xenobiotic response element (XRE)-like element (XREL1) located at -712 and two additional XRE-like elements located at +755 (XREL2) and +850 (XREL3). Functional analysis using luciferase assay showed that XREL1, XREL2, and XREL3 are all inducible by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin treatment, with XREL2 being the most potent.

More recently, it was reported that the Nrf2 expression could also be regulated in a reoxygenation-specific manner (Leonard et al., 2006). It was also shown that there was a nuclear accumulation of Nrf2 in response to reoxygenation during ischemic reperfusion injury. Further more, ROS during reoxygenation using NAC resulted in inhibition of Nrf-2 activation. (Leonard et al., 2006). Thus, although it appears that redox state of a cell is a major regulator of Nrf2 expression; several other factors as described above also modulate the expression of Nrf2. It is possible that new biochemical factors may be identified in future, which may also play a role in the regulation of expression of Nrf2 gene.

4.2. Nrf2 in GSH biosynthesis in response to inhaled oxidants

Nrf1 and Nrf2, which are important in the transcriptional regulation of human and mouse GCL subunit expressions and activity and in regulating cellular GSH levels. The ARE present in the human GCLC and GCLM promoters is trans-activated by Nrf2 in response to α -naphthoflavone, pyrrolidine dithiocarbamate, and *tert*-butylhydroquinone (TBH) and other exogenous agents (Erikson et al., 2002). Although Nrf1 knockout mice have been reported to die *in utero*, the hepatocytes from the fetus and the embryonal fibroblasts from these animals

have lower GSH levels and are more susceptible to oxidative stress than wild-type (WT) mice (Chen et al., 2003; Kwong et al., 1999). Lower GSH levels have been observed in mice deficient in Nrf2 and are found to be more susceptible to acetaminophen-induced liver injury compared to their WT counterparts (Chan et al., 2001). Whereas, Nrf1 and Nrf2 knockout mice exhibit lower GCLC expression (Chan et al., 2001, Chen et al., 2003), over-expression of the same has been shown to induce the human GCLC promoter activity (Wild et al., 1999). Interestingly, TBH also induces expression of rat GCLC and enhances activity of the rat GCLC promoter even in absence of ARE in the promoter region of GCLC (Yang et al., 2002; Yang et al., 2001). In such a situation it appears that TBH mediated induction of GCLC is essentially mediated by AP-1 (Yang et al., 2002).

Nrf2 mediated induction of human GCLM and GCLC promoters has been reported to be a result of trans-activation via Jun or Maf proteins (Erickson et al., 2002.). The role of Nrf2 in GCL expression has been further established using Nrf2 knockout mice, which demonstrated increased susceptibility to oxidant/acetaminophen-induced lung/liver injury, a phenomenon that was attributed to decreased GCL expression and GSH levels (Chan et al., 2001; Reddy et al., 2007). Over-expression of Nrf2 increased human GCLC and GCLM promoter activity (Jeyapaul and Jaiswal, 2000; Wild et al., 1999) and restored GCL subunit expression and GSH levels in Nrf2 knockout fibroblasts (Chan and Kwong 2000). A similar effect has been also reported in Nrf1 knockout rat fetal hepatocytes, where lower GCLC expression was observed (Chen et al., 2003). Nrf1 over-expression was also found to induce the human GCLC promoter activity via ARE (Jeyapaul and Jaiswal, 2000), an effect that is also reported for the mouse GCLC and GCLM promoters (Lee et al., 2003).

Human and rat GCL subunits might have similar regulatory mechanisms (Lu, 1999). It is of interest to note that although the 1.8-kb 5' flanking region of the rat GCLC does not contain any consensus ARE element, the reporter activity however, driven by a recombinant rat GCLC-luciferase construct is induced by TBH treatment (Yang et al., 2002). A time-dependent induction in the expression of both genes was recorded in response to TBH. Although TBH treatment induced rat GCLC promoter activity in a wide variety of cell types, however, the induction levels were different in Nrf2 knockout and Nrf1 knockout cell types (Yang et al., 2005). It was previously shown that the rat GCLC promoter is induced by treatment with acetaldehyde and TBH (Yang et al., 2001 and 2002). Consensus NF- κ B and AP-1 binding sites are present in the rat GCLC -595/+2 promoter fragment (Yang et al., 2001) and the nuclear binding of the two factors was found to be positive after treatment of cells with acetaldehyde (Yang et al., 2001).

In F1 fibroblast cells (Nrf1 knockout) and F2 fibroblast cells (Nrf2 knockout) the AP-1 family members, c-Fos and c-Jun levels were found to be decreased by upto 70 to 85%, JunB levels did not show any change. While JunD levels were found to be unchanged in F1 cells, slightly decreased levels were reported in F2 cells. The Fos related antigen (Fra), Fra-2 levels were slightly increased in F1 cells, and Fra-1 and Jun activating binding protein-1 (JAB-1) levels were reported to be markedly induced, especially in F2 cells (Yang et al., 2005). Among the NF- κ B family members, p50 and p65 were shown to be reduced by upto 60 to 70%, RelB levels were slightly decreased and c-Rel levels were reported to be induced. The molecular mechanism underlying the alterations in the protein levels of Fra-1, p50, and p65, c-Jun, c-Fos is possibly at the transcriptional level, as mRNA levels of these genes were found to be decreased (increased in the case of Fra-1) in F1 and F2 cells when compared to WT cells.

The above alterations significantly decreased the TBH dependent nuclear binding of AP-1 and NF- κ B. However, transfection of F2 cells with Nrf2 expression vector interestingly increased the base line levels of p50, p65, c-Jun, GCLC and c-Fos, decreased expression of Fra-1, and restored the cell response to TBH. Thus it appears that Nrf2 is intimately related to the

expression of the aforementioned genes and may be an important regulator of GCLC and GCLM promoter activation and synthesis of GSH (Yang et al., 2005). In a recent report the role of Nrf2 in biosynthesis of GSH has been confirmed wherein it was shown that resveratrol, a red wine polyphenol induces GSH synthesis via activation of Nrf2 in human lung epithelial cells (Biswas et al., 2005; Kode et al., 2008).

5. Therapies/clinical trials with GSH analogs

Extracellular augmentation of GSH has been tried through intravenous administration of GSH, oral ingestion of GSH, and aerosol inhalation of nebulized GSH in an attempt to reduce inflammation in various lung diseases (Rahman et al., 2006; Rahman and MacNee, 1999). Various GSH analogues and substitutes are in vogue for therapeutic application (Figure 2) in various respiratory diseases which are described below:

5.1. N-acetyl-L-cysteine (NAC)

The free thiol group of NAC is capable of interacting with the electrophilic group of free radicals thereby forming NAC thiol, with NAC disulphide or NAC conjugates as a major end product (Cotgreave, 1997). The availability of cysteine for GSH is a fundamental factor in the regulation of GSH production. Radioactivity associated with administration of ^{14}C -NAC distributed to most tissues examined within 1 hour of administration with peak radioactivity levels occurring within 1–4 hours and for a majority of the tissues examined, radioactivity remained elevated for up to 12 hours or more (Arfsten et al., 2007). The results of this study demonstrated the possibility that NAC could provide some benefit in preventing or reducing toxicity related to exposure to chemical irritants in some tissues by increasing tissue NAC and/or cysteine levels, GSH concentrations, and GST activity. However, the finding of this study that GxP activity is elevated, albeit transiently, following repeat administration of NAC suggests that repeated administration of NAC may induce oxidative stress in some tissues and further studies are needed to confirm this finding (Arfsten et al., 2007).

NAC may also reduce cystine to cysteine, which is an important mechanism for intracellular GSH elevation in vivo in lungs (Burgunder et al., 1989). It not only reduces disulphide bonds (a property of a good reducing agent), but also has the potential to interact directly with oxidants. NAC is also used as a mucolytic agent, to reduce mucus viscosity and to improve mucociliary clearance. A number of systematic reviews have evaluated the effects of treatment with mucolytic agents in patients with COPD (Grandjean et al, 2000; Dekhuijzen, 2004). Mucolytic treatment was associated with a significant reduction of 0.79 exacerbations per patient per year compared to placebo, a 29% decrease (Table 3) (Grandjean et al, 2000; Dekhuijzen, 2004; Decramer et al., 2001; Decramer et al., 2005; Poole and Black, 2003). Although, how mucolytic agents work is still unknown, they may reduce exacerbations by altering mucus production, by breakdown of sulphhydryl group, or through antibacterial or immunostimulatory effects (Poole and Black, 2001 and 2003). Pharmacological administration of NAC has been used in an attempt to enhance lung GSH in patients with COPD with varying success (Bridgemen et al., 1994; Rasmussen and Glennow 1988). Van Schooten et al. (Van Schooten et al., 2002) reported in a randomized, double-blind, placebo-controlled phase II trial that a 6-month oral dose of 600 mg b.i.d. reduced various plasma and BALF oxidative biomarkers in smokers. A recent report has suggested that NAC attenuates lung ischemia-reperfusion injury after lung transplantation (Inci et al., 2007). In addition, on the mechanistic front, NAC has been shown to attenuate allergic lung disease by regulating the activation of NF- κ B and hypoxia-inducible factor-1 (HIF-1) (Lee et al., 2007) and also attenuates acute lung injury caused due to phorbol myristate acetate in isolated rat lungs (Chuang et al., 2007).

Similarly, it has been shown that treatment with NAC (600 mg once daily for 12 months) also reduce the concentration of H_2O_2 in exhaled breath condensate compared with placebo in stable

COPD patients (Kasielski and Nowak, 2001). Another recent clinical trial also proved that oral administration of NAC 600 mg b.i.d. for 2 months rapidly reduces the H₂O₂ in airways of stable COPD patients (De Benedetto et al., 2005). This study was mainly aimed at determining the utility of H₂O₂ as a tool for assessment and monitoring of oxidative stress in COPD. The mechanism for this effect of NAC on H₂O₂ is not known but presumably NAC acts as a sink for ROS, which are increased in these patients (Rahman, 2005). However, a multicentre study using NAC delivered by metered dose inhalers in patients with chronic cough failed to show a positive effect on wellbeing, sensation of dyspnoea, cough or lung function (Dueholm et al., 1992). Recently, NAC has been shown to downregulate inflammatory responses in CF patients (Tirouvanziam et al., 2006)

Whilst there is some evidence that the administration of NAC provides benefit for some COPD patients, it is not clear whether this could be used as maintenance therapy (Decramer et al., 2001). A phase III multicentre Bronchitis Randomized on NAC Cost- Utility Study (BRONCUS) has recently been completed, with the aim of addressing this question and determining whether the effectiveness of NAC as an “antioxidant” results in an alteration in the rate of decline in FEV₁, exacerbation rate and quality of life (QoL) in patients with moderate-to-severe COPD (Gerrits et al., 2003) (Table 3). The results of this trial showed no effect on decline in FEV₁, but a reduction in lung overinflation in patients with severe COPD and in exacerbation rate in patients who were not treated with inhaled glucocorticoids (Decramer et al., 2005). The variability in all the current studies using NAC 600 mg p.o. q.d. i.e. orally once a day may simply reflect the fact that the dose is not high enough. Thus, further studies are required at higher doses (1,200 or 1,800 mg/day) or using other thiol agents with greater bioavailability in order to observe any clinical benefit on lung function, reduced exacerbation rate and improved health status. IPF is associated with reduced pulmonary GSH and oxidative stress (Beeh et al., 2002; Rahman et al., 1999a). Ongoing clinical trials with NAC in these patients suggest that this thiol compound is well tolerated and may increase pulmonary levels of GSH.

5.1.1. Pharmacology and Pharmacokinetics of NAC—Following an oral dose of 600 mg, NAC is rapidly absorbed with a peak of 4.6 μM after 60 min (Tsikas et al., 1998), the plasma half-life being 2.5 hours. Following oral administration, reduced NAC has a terminal half-life of 6.25h (Holdiness, 1991). No NAC is detectable 10–12 hours after administration (De Caro et al., 1989). It has been estimated that the oral bioavailability of the intact NAC molecule was about 10% (Borgstrom and Kagedal, 1990). NAC when orally administered is metabolized into another compounds since accompanying increase in non-protein and protein sulphhydryl groups were found in plasma (Cotgreave et al., 1987; De Caro et al., 1989). It is important to note that increasing the dose also increased NAC bioavailability and time for maximal plasma concentration (Borgstrom and Kagedal, 1990) and higher concentration in plasma may be achieved after intravenous administration (Crouch and Rusho, 2005). The volume of distribution ranges from 0.33 to 0.47 L/kg and protein binding is significant, reaching approximately 50% 4 hours post-administration (Holdiness, 1991). Pharmacokinetic information is not available regarding the penetrability of NAC across the blood-brain barrier or placenta, or into breast milk. Whereas, renal clearance has been reported as 0.190 to 0.211 L/h/kg, up to approximately 70% of the total body clearance is non-renal. Post-absorption, NAC is rapidly metabolized to cysteine, which has reducing and antioxidant properties due to the presence of the thiol group and also is a direct precursor of the GSH. Because of its ability to reduce disulfide bounds it is widely used to reduce viscosity and elasticity of the mucus (Aruoma et al., 1989). In order to exert some effects in the lungs, concentration of NAC or its derivatives have to be sufficiently elevated in bronchial epithelium or in ELF. Nevertheless, no NAC was found in BALF after 2-weeks of NAC intake (200 mg t.i.d) by healthy volunteers (Cotgreave et al., 1987). The details of pharmacology and pharmacokinetics of NAC *in vivo*

and its implications in patients with COPD is nicely reviewed by Sadowska et al. (Sadowska et al., 2007).

5.2. N-acetylcystein

N-acetylcystein (NAL), a lysine salt of NAC, has a neutral pH in solution, whereas NAC is acidic but retains the mucolytic and antioxidant (reducing) actions of NAC. NAL can be aerosolised into the lung without causing significant side effects (Gillissen et al., 1997); Antonicelli et al. (Antonicelli et al., 2002) compared the effect of NAL and NAC in vitro and found that both drugs enhance intracellular GSH in alveolar epithelial cells and inhibited H₂O₂ and O₂ N-released from human blood-derived neutrophils from smokers with COPD. NAL also inhibited ROS generation induced by serum-opsonized zymosan by human polymorphonuclear neutrophils. This in vitro inhibitory response was comparable to the effects of NAC (Ekberg-Jansson et al., 1999). Antonicelli et al. (Antonicelli et al., 2002) have shown that NAL inhibited oxidant-mediated IL-8 release in alveolar epithelial A549 cells suggesting an anti-inflammatory effect of NAL. Therefore, NAL may represent an interesting alternative approach to augment the antioxidant screen and thereby inhibiting inflammatory responses in the lungs and can be administered by inhalation.

5.3. N-isobutyrylcysteine

Since NAC becomes hydrolyzed in biological systems, the measured bioavailability of the drug is low. Thus, it was speculated that a drug might be synthesized that possessed greater bioavailability than NAC, and could be used as a more effective treatment for COPD. N-isobutyrylcysteine (NIC) is a NAC-like thiol compound that does not undergo effective first pass hydrolysis and hence has a higher oral bioavailability than NAC (Ekberg-Jansson et al., 1999). The oral bioavailability can be as high as 80%, dependent on food intake. However, when evaluated as a therapy for exacerbations of chronic bronchitis, NIC performed no better than placebo, and not as well as NAC (Gillissen et al., 1997). Recently, a study of NIC also failed to reduce exacerbation rates in patients with COPD (Ekberg-Jansson et al., 1999).

5.4. Erdosteine

Erdosteine is a new thiol compound that also acts as an antioxidant, but in addition has mucoactive properties and reduces bacterial adhesiveness (Moretti and Marchioni, 2007). In the "EQUALIFE" randomized placebo-controlled clinical study, erdosteine was dosed orally 300 mg b.i.d. for 8 months (Moretti et al., 2004). Patients receiving erdosteine had significantly fewer exacerbations and spent less days in hospital than the placebo group (Moretti et al., 2004). Moreover, patients receiving erdosteine showed no reduction in lung function over this period and a significant improvement in health-related QoL (Moretti et al., 2004). It is not clear whether clinical benefit is due to its antioxidant or mucolytic actions since the findings are consistent with the Cochrane review meta-analysis on mucolytic reagents in chronic bronchitis (Stey et al., 2000). It is possible that erdosteine may reduce bacterial colonization through a direct effect on adhesion (Moretti et al., 2004). A clinical trial on the combination of steroids and erdosteine in patients with COPD is needed.

5.5. Fudosteine

Fudosteine, [(–)-(R)-2-amino-3-(3-hydroxypropylthio)] propionic acid, has been used as mucoactive agent with indications for chronic respiratory diseases such as bronchial asthma, chronic bronchitis, pulmonary emphysema, COPD, bronchiectasis, pulmonary tuberculosis, pneumoconiosis, atypical mycobacterial disease, and diffuse panbronchiolitis (Komatsu et al., 2005). Fudosteine are a cysteine donating compounds that increase the cysteine levels of the cells and have greater bioavailability than NAC. These thiol compounds are well tolerated and have been shown to increase mitochondrial levels of GSH in alveolar type II cells (Guidot and

Brown, 2000). GSH esters, particularly GSH monoethyl esters can increase the GSH levels of these cells by cleavage of an ester bond (an ethyl group esterified to glycine). GSH esters have been shown to increase GSH levels in the lungs of rats; however, this compound can be cytotoxic and variation in the uptake levels of GSH has been shown in various cellular models (Butterworth et al., 1993). Fudosteine inhibits MUC5AC mucin hypersecretion by reducing the expression of the MUC5AC gene (Rhee et al., 2008). Although EGFR is a common molecule for the activation of MUC5AC gene expression, the inhibitory effect of fudosteine is not related to EGFR. With respect to the MUC5AC mucin secretion pathways, fudosteine inhibited the phosphorylation of ERK1/2 and p38 MAPK in LPS-treated rats and of ERK1/2 in TNF- α -treated NCI-H292 cells (Rhee et al., 2008). These findings suggest that fudosteine may be useful in controlling stress-related mucus secretion states in patients with asthma, bronchiectasis or COPD.

5.6. Carbocysteine

S-carboxymethylcysteine (carbocysteine or S-CMC) is a muco-active drug with *in vitro* free radical scavenging and anti-inflammatory properties. Carbocysteine is available as an oral preparation both as S-CMC and its lysine salt (S-CMC-lys). The lysine group is cleaved on gastric absorption to form the active drug S-CMC. Carbocysteine is well absorbed when taken orally and peak serum concentrations are achieved at 1–1.7 hours and the plasma half life is 1.33 hours. It achieves good translocation into lung tissue and bronchial secretions (Braga et al., 1982). Approximately 30–60% of the drug is excreted unchanged in urine. There is evidence from animal models that carbocysteine increases chloride transport across the airway epithelium and this may also contribute to its muco-regulatory action (Colombo et al., 1994). Evidence from animal models of respiratory epithelial repair demonstrates that carbocysteine reverses neutral endopeptidase levels and cough sensitivity (but not methacholine-induced bronchoconstriction) in rodents with antigen-damaged tracheal epithelium in a dose-related manner (Katayama et al., 2001). Animal studies have demonstrated the anti-inflammatory action of carbocysteine in models of pulmonary inflammation involving several different cytokine profiles. Oral pre-treatment with S-CMC-lys appears to attenuate neutrophil recruitment in acute IL-1 β -induced airway inflammation and neutrophil, macrophage and eosinophil migration to the pleural space in carrageenan-induced pleurisy (Asti et al., 1995). Administration of carbocysteine to rats with sulfur dioxide-induced airway inflammation attenuates the secretion of abnormal mucous glycoproteins and reduces inflammatory cells, free radical and elastase activity in their BALF (Ishibashi et al., 2001). Carbocysteine has been clinically used to treat patients with COPD. A recent PEACE study has revealed that the COPD patients treated with carbocysteine experienced decreased numbers of exacerbations per year (Zheng et al., 2008). Non-significant interactions were found between the preventive effects and COPD severity, smoking, as well as concomitant use of inhaled corticosteroids. Furthermore, carbocysteine (carbocisteine) was well-tolerated.

5.7. GSH peroxidase mimetic

Small molecules with enzymatic activity similar to GPx have been developed, such as the seleno-organic compound ebselen. Selenium is an important element in the GPx catalysis of the reaction between GSH and ROS. Consequently, ebselen increases the efficiency of GSH as an antioxidant, and thus may be used as a therapy against oxidative stress and inflammation. Recent studies have shown that ebselen inhibits airway inflammation (neutrophil recruitment and chemokine expression) in response to LPS in various animal models (Haddad et al., 2002; Zhang et al., 2002). It would be interesting to see whether similar results can be obtained by ebselen in inhibiting the airway inflammation of cigarette smokers.

6. GSH and lung diseases

The lung being directly exposed to the air is susceptible to attack from airborne materials, cigarette smoke, environmental oxidants and other pollutants and toxins (Kidd, 1985). GSH and GSH-associated enzymes present in the lower respiratory tract are believed to act as a first line of defense against such attacks by external agents (Deleve and Kaplowitz, 1990; Pacht et al., 1991). Sustained oxidative challenge leads to depletion of lung GSH along with other antioxidants.

GSH deficiencies have now been documented in number of lung diseases, which include ARDS, COPD, asthma, CF, IPF and neonatal lung disease (Lomaestro and Malone, 1995). ARDS patients having sepsis have been found to be deficient in GSH in the ELF compared to healthy subjects (Bunnell and Pacht, 1993) and a large percentage of GSH is in its oxidized, GSSG form. This is indicative of an oxidative stress in the lower respiratory tract. Treatment with NAC to such patients significantly decreased the time spent in the hospital (Suter et al., 1994). Asthmatics also demonstrate increased ROS generation during airway inflammation. Patients with mild form of asthma have a capacity to adaptive increases in their lung alveolar GSH levels (Smith et al., 1993). IPF patients however, the GSH levels are only about 25% of the normal levels, which is the cause of the pathophysiology underlying the development and progression of this disease (Deleve and Kaplowitz, 1990).

Infants born pre-maturely at 25 weeks of gestational age have been found to have decreased levels of GSH in the lungs compared to those who are born at 40 weeks (Grigg et al, 1993). Infants born at 35-weeks also showed lower levels of lung GSH and particularly susceptible to chronic lung diseases later in their lives. Thus many lung diseases are related to the status of GSH in the lungs and lung compartments.

7. Future Perspectives

Therapies with GSH and its analogues as discussed above have been widely practiced and studied. Several clinical trials have also been undertaken (Table 3). Most of these studies have revealed that such a mode of treatment may not necessarily have positive outcome. Several studies have reported undesirable effects suggesting that direct GSH therapy may not be an appropriate way of increasing GSH levels in lung ELF and cells in COPD. The bioavailability of GSH, pH, osmolality in the inflammatory micro-environment, and the resultant formation of toxic products (GSSG and GSH-adducts) are further challenges for direct GSH administration. Alternative formulations may address bioavailability, such as liposomal delivery, but at present it seems that direct administration of GSH will not be successful in treating COPD. Increasing the activity of GCL would also be expected to increase cellular GSH levels, and induction of GCL by pharmacological agents also holds great promise in protection against chronic inflammation and oxidant-mediated injury in COPD. Given the critical role of the GSH pathway in the detoxification of many drugs and xenobiotics, the reported differences in basal tissue distribution among mouse, rat and canine has far-reaching implications in comparing responses of these species in safety testing of GSH and or its analogues as therapeutic agents (Mattes et al., 2006).

Repeated administration of NAC may induce oxidative stress in some tissues and further studies are needed to confirm this finding (Arfsten et al., 2007). NAC may also reduce cystine to cysteine, which is an important mechanism for intracellular GSH elevation in vivo in lungs (Burgunder et al., 1989). It not only reduces disulphide bonds (a property of a good reducing agent), but also has the potential to interact directly with oxidants. Since NAC becomes hydrolyzed in biological systems, the measured bioavailability of the drug is low. This aspect also requires attention for further research. Similarly, it is not clear whether clinical benefit of erdosteine, fudosteine and carbocysteine is due to its antioxidant or mucolytic actions which

are consistent with the Cochrane review meta-analysis on mucolytic reagents in chronic bronchitis (Stey et al., 2000). Overall, it may be pertinent to suggest that therapeutic research involving GSH or its analogues or thiol compounds must be carefully designed keeping in mind the species barrier, bioavailability, half life of the analogues, and generation of toxic byproducts by the analogues and interference with metabolic and signaling pathways in a cell or tissue. Since patients may have different level of antioxidants in them and since the oxidative states may differ considerably depending on nutritional, behavioral and occupational states, proper understanding of the above issues may lead to development of better thiol/GSH-dependent therapeutic strategies for pulmonary diseases.

Acknowledgments

This work was supported by the National Institutes of Health (NIH) R01-HL085613 and National Institute of Environmental Health Sciences Center (NIEHS) Grant ES-01247.

This work was supported by the National Institutes of Health (NIH)-NHLBI R01-HL085613 and National Institute of Environmental Health Sciences Center (NIEHS) Grant ES-01247.

Abbreviations

AP-1	Activator Protein-1
ARDS	Acute respiratory distress syndrome
ARE	Antioxidant response element
BALF	Bronchoalveolar Lavage Fluid
CF	Cystic Fibrosis
COPD	Chronic Obstructive Pulmonary Disease
EGF	Epidermal growth factor
ELF	Epithelial Lining Fluid
EpRE	Electrophilic Response Element
ER	Endoplasmic reticulum
ERK	extracellular regulated protein kinase
γ-GT	γ -glutamyl transpeptidase
GCL	Glutamate Cysteine Ligase

GCLC	Glutamate-Cysteine Ligase, Catalytic subunit
GCLM	Glutamate-Cysteine Ligase, Modifying subunit
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione Disulfide
H₂O₂	Hydrogen peroxide
IL-8	Interleukin-8
IL-13	Interleukin-13
IPF	Idiopathic pulmonary fibrosis
MAPK	Mitogen activated protein kinase
NAC	N-acetyl-L-cysteine
NAL	N-acetylcystein
NF-κB	Nuclear Factor-kappaB
NO₂	Nitrogen dioxide
NO•	Nitric Oxide
Nrf	cap 'n' collar-basic leucine zipper (CNC-bZIP) family protein or nuclear redox factor
•OH	Hydroxyl radical
ONOO-	Peroxynitrite
ROS	Reactive Oxygen Species
RNS	

Reactive Nitrogen Species

TGF- β 1Transforming Growth Factor- β 1**TNF- α**

Tumor Necrosis Factor-alpha

Trx

Thioredoxin

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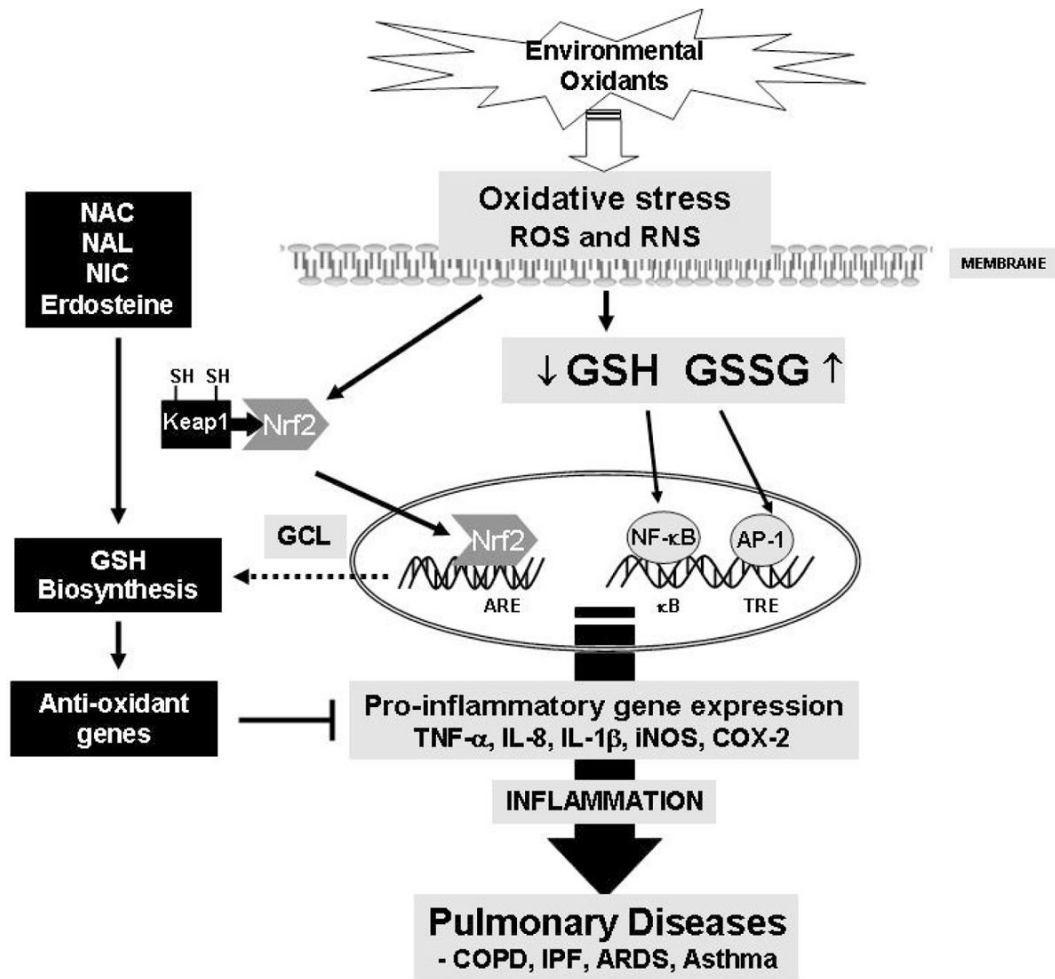
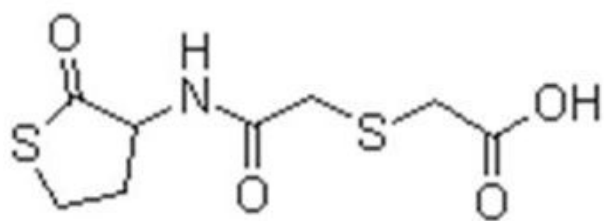
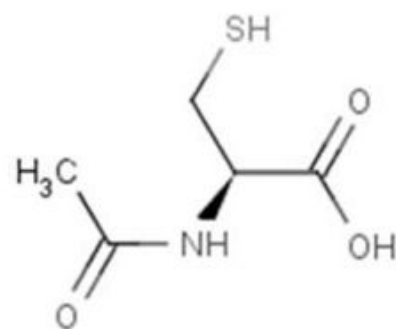


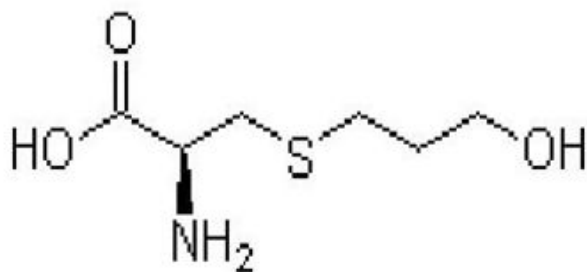
Fig. 1. Oxidative alteration in the intracellular redox ratio of GSH/GSSG levels and redox regulation of pro-inflammatory and anti-inflammatory diseases. Oxidative alteration in the intracellular redox ratio of GSH/GSSG levels by environmental oxidants can modulate NF- κ B and AP-1 activation leading to transcription of pro-inflammatory genes. The expression of pro-inflammatory mediators would lead to chronic inflammation which is involved in the pathogenesis of various pulmonary diseases, such as COPD, IPF, ARDS, and asthma. On the other hand during oxidative stress the ARE present in the human GCLC and GCLM promoters is trans-activated by Nrf2 due to alteration in GSH and GSSG ratio leading to synthesis of GSH. The imbalance of antioxidant and pro-inflammatory genes can lead to chronic inflammation. Various thiol compounds, such as N-acetyl-L-cysteine (NAC), N-acetylcystein (NAL), N-isobutyrylcysteine (NIC) and erdosteine can supply cysteine for GSH biosynthesis.



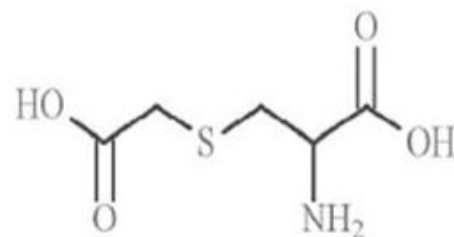
Erdosteine



N- Acetylcysteine



Fudosteine



Carbocysteine

Fig. 2.
Chemical structures of key thiol compounds in clinical trials.

Table 1

Levels of intra-cellular GSH in different cell types

Cell type	Basal GSH level (nmol/mg protein)
Human alveolar epithelial cells, A549	150
Human primary airway epithelial cells	60
Human hepatocarcinoma, HepG2	71
Bovine arterial endothelial cells	20
Rat lung epithelial, L2	18
Human umbilical vein endothelial cells	4
Human monocytes/macrophages (MonoMac Cells)	10

Table 2
Epithelial lining fluid GSH concentration in inflammatory lung diseases

	Concentration (μM)	
	Control	Patients
Controls (non-smokers)	339 \pm 112	-
Smokers	544 \pm 97.6	-
Idiopathic pulmonary fibrosis	429 \pm 34	97.0 \pm 18
Acute respiratory distress syndrome	651 \pm 103.1	31.5 \pm 8.4
Lung allograft	302.6 \pm 40.8	94.0 \pm 9.7
Cystic fibrosis	257 \pm 21	78.0 \pm 13
HIV-seropositive	245 \pm 12	170 \pm 23
Asthma ($\mu\text{M}/\text{mg}$ protein)	23.3 \pm 3	36.5 \pm 9.4
COPD (stable) (nmol/mg protein)	4	10
COPD (during exacerbations) (nmol/mg protein)	4	2

Table 3
Clinical Trials conducted for the efficacy of thiols in smokers and COPD subjects

SN	Name of Trial	Antioxidant used	Aim of Study	Disease	Outcome
1	BRONCUS	NAC	Effect on FEV ₁	COPD	No effect. A reduction in lung over inflation in patients with severe COPD without inhaled glucocorticoids
2	Systematic Cochrane review of 23 randomized controlled trials	NAC (2 months of oral therapy)	Effect of NAC and antibiotics on number of days of disability	COPD	No effect on lung function. 29% decrease in exacerbations. Good reduction in days of disability (0.85 days/patient/month)
3	Systematic Cochrane review of randomized controlled trials of 11 out of 39 reviewed trials	NAC	Used a validated score for evaluation of quality of each study	COPD	9 trials showed prevention of exacerbations and 5 showed improvement in symptoms compared to 34.6% patients receiving placebos
4	A meta analysis of published trials	NAC (600 mg bid for 12 months)	To assess the possible prophylactic benefit of prolonged treatment Effect on H ₂ O ₂ and TBARS in EBC	COPD COPD	23% decrease in acute exacerbation No change in TBARS, reduced H ₂ O ₂
5	EQUALIFE studies	Vecrine-erdosteine, thiol compound, 300mg bid for months	Exacerbation rate, hospitalization, lung function and quality of life in 124 patients	COPD	Decreased exacerbations and fewer days in hospital. No loss of lung function and improvement in health-related quality of life
6	PEACE study	Carboeysteine (carbocysteine)	Effect on rate of exacerbations	COPD	Long-term (one year) use of carbocysteine (1500 mg/day) produced reduction in numbers of exacerbations in patients with COPD

BRONCUS = Bronchitis Randomized on N-acetyl-L-cysteine Cost Utility Study, FEV₁ = Forced expiratory volume in 1 second, TBARS = Thiobarbituric acid reactive substances, NAC = N-acetyl-L-cysteine, EBC = Exhaled Breath Condensate.