Quantum Proteolysis by Neutrophils*

Implications for Pulmonary Emphysema in α₁-Antitrypsin Deficiency

Edward J. Campbell, MD; M. A. Campbell, MT(ASCP); S. S. Boukedes, BS; and Caroline A. Owen, MD, PhD

References


Quantum Proteolysis by Neutrophils*

Implications for Pulmonary Emphysema in α₁-Antitrypsin Deficiency

Edward J. Campbell, MD; M. A. Campbell, MT(ASCP); S. S. Boukedes, BS; and Caroline A. Owen, MD, PhD

References


Quantum Proteolysis by Neutrophils*

Implications for Pulmonary Emphysema in α₁-Antitrypsin Deficiency

Edward J. Campbell, MD; M. A. Campbell, MT(ASCP); S. S. Boukedes, BS; and Caroline A. Owen, MD, PhD

References


Oxidants/Antioxidants and COPD*

William MacNee, MD

(CHEST 2000; 117:303S–317S)

Oxidative stress results from an oxidant/antioxidant imbalance, an excess of oxidants and/or a depletion of antioxidants. Oxidative stress is thought to play an important role in the pathogenesis of a number of lung diseases, not only through direct injurious effects, but by involvement in the molecular mechanisms that control lung inflammation. A number of studies have shown an increased oxidant burden and consequently increased markers of oxidative stress in the airspaces, breath, blood, and urine in smokers and in patients with COPD. The presence of oxidative stress has important consequences for the pathogenesis of COPD. These include oxidative inactivation of antiproteinases, airspace epithelial injury,

*From the Edinburgh Lung Environmental Group Initiative, Colt Research Laboratories, University of Edinburgh, Edinburgh, Scotland, UK.

Correspondence to: W. MacNee, MD, Respiratory Medicine, Edinburgh Lung Environmental Group Initiative, Colt Research Laboratories, Wilkie Building, Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland, UK; e-mail: w.macnee@ed.ac.uk

Abbreviation: AAT = α₁-antitrypsin

α₁-Antitrypsin (AAT) deficiency is the most prevalent potentially fatal hereditary disease in white individuals, and is an important risk factor for pulmonary emphysema, especially in cigarette smokers. Traditional enzyme kinetics provide a poor explanation for the increased risk of lung injury in AAT deficiency. We have found that when millimolar concentrations of leukocyte elastase are released from single azurophil granules of activated neutrophils, they transiently overwhelm local proteinase inhibitors, leading to evanescent quantum bursts of proteolytic activity. Catalysis is quenched when enzyme concentration no longer exceeds that of pericellular inhibitors. 1,2 Herein, we tested the possibility that quantum proteolytic events are abnormal in AAT deficiency. We incubated neutrophils on opsonized fluoresceinated fibronectin in serum from individuals with various AAT phenotypes, and then measured and modeled quantum proteolytic events. The mean areas of the events in serum from heterozygotes (Pi MZ and Pi SZ) were 16.1 ± (SEM) 4.0 μm² and 14.2 ± 3.3 μm², respectively, which were slightly (but significantly) larger than those in serum from normals (Pi M), which were 9.7 ± 1.2 μm². In marked contrast, events in serum from AAT-deficient individuals were 97.4 ± 7.8 μm². Diffusion modeling predicted that local elastase concentrations exceed AAT concentrations for <20 ms and >80 ms in Pi M and Pi Z individuals, respectively. Thus, quantum proteolytic events are abnormally large and prolonged in AAT deficiency, leading directly to an increased risk of tissue injury in the immediate vicinity of activated neutrophils. These results have potentially important implications for the pathogenesis and prevention of lung disease in AAT deficiency.
increased sequestration of neutrophils in the pulmonary microvasculature, and gene expression of proinflammatory mediators. With regard to the latter, oxidative stress has a role in enhancing the inflammation that occurs in smokers and patients with COPD, through the activation of redox-sensitive transcription factors such as nuclear factor-κB and activator protein-1, which regulate the genes for proinflammatory mediators and protective antioxidant gene expression.

The sources of the increased oxidative stress in patients with COPD are derived from the increased burden of oxidants present in cigarette smoke, or from the increased amounts of reactive oxygen species released from leukocytes, both in the airspaces and in the blood. Antioxidant depletion or deficiency in antioxidants may contribute to oxidative stress. The development of airflow limitation is related to dietary deficiency of antioxidants, and hence dietary supplementation may be a beneficial therapeutic intervention in this condition. Antioxidants that have good bioavailability or molecules that have antioxidant enzyme activity may be therapies that not only protect against the direct injurious effects of oxidants, but may fundamentally alter the inflammatory events that play an important part in the pathogenesis of COPD.

Key words: antioxidant; COPD; oxidants; reactive oxygen species

Abbreviations: α1-AT = α1-antitrypsin; AP = activator protein; BALF = BAL fluid; CSC = cigarette smoke condensate; ELF = epithelial lining fluid; γ-GCS = γ-glutamylcysteine synthetase; GP = glutathione peroxidase; GSH = glutathione; IL = interleukin; NAC = N-acetylcysteine; NAL = N-acetylcysteine; NF-κB = nuclear factor-κB; NO = nitric oxide; O2− = superoxide anion; ROS = reactive oxygen species; RTLF = respiratory tract lining fluid; SOD = superoxide dismutase; TBARS = thiobarbituric acid reactive substances; TNF = tumor necrosis factor

COPD is a slowly progressive condition characterized by airflow limitation, which is largely irreversible. A smoking history of at least 20 pack-years is usual, reflecting the fact that smoking is the main etiologic factor in this condition, which far outweighs any of the other risk factors. The pathogenesis of COPD is therefore strongly linked to the effects of cigarette smoke. Since cigarette smoke contains 10¹⁷ molecules per puff, it has been proposed that an oxidant/antioxidant imbalance occurs in smokers, and an increased oxidant burden in smokers and patients with COPD.

Oxidants in Cigarette Smoke

Cigarette smoke is a complex mixture of > 4,700 chemical compounds of which free radicals and other oxidants are present in high concentrations. Free radicals are present in both the tar and the gas phases of cigarette smoke. The gas phase of cigarette smoke contains approximately 10⁶ radicals per puff, primarily of the alkyl and peroxyl types. Nitric oxide (NO) is another oxidant that is present in cigarette smoke in concentrations of 500 to 1,000 ppm. NO reacts quickly with the superoxide anion (O2−) to form peroxynitrite, and with peroxyl radicals to give alkyl peroxynitrites.

The tar phase of cigarette contains more stable radicals, such as the semiquinone radical, which can react with oxygen to produce O2−, the hydroxyl radical, and hydrogen peroxide. The tar phase is also an effective metal chelator and can bind iron to produce the tar-semiquinone + tar-Fe²⁺, which can generate hydrogen peroxide.

The epithelial lining fluid (ELF) and mucous are the first line of defense in the lungs against inhaled oxidants by quenching the short-lived radicals in the gas phase of cigarette smoke. However, cigarette smoke condensate (CSC), which forms in the ELF, may continue to produce reactive oxygen species (ROS) for a considerable period in patients with COPD, as part of the pathogenesis of this condition.

Cell-Derived Oxidants

The direct increase in the oxidative burden produced by inhaling cigarette smoke can be further enhanced in smokers’ lungs by the release of oxygen radicals from inflammatory leukocytes, both neutrophils and macrophages, which are known to migrate into the lungs of cigarette smokers. Increased amounts of oxidants such as O2− and hydrogen peroxide are released from the leukocytes of smokers, compared with those from nonsmokers.

Iron is a critical element in many oxidative reactions. The generation of oxidants in ELF in smokers is further enhanced by the presence of increased amounts of free iron in the airspaces. The intracellular iron content of alveolar macrophages is increased in cigarette smokers and is increased further in those who develop chronic bronchitis, compared with nonsmokers. Furthermore, macrophages from smokers release more free iron in vitro than those from nonsmokers.

Free iron in the ferrous form can take part in the Fenton and Haber-Weiss reactions, which generate the hydroxyl radical, a free radical that is extremely damaging to all tissues, particularly to cell membranes, producing lipid peroxidation.

Direct oxidative damage to components of the lung matrix (such as elastin and collagen) can result from oxidants in cigarette smoke. Elastin synthesis and repair can also be impaired by cigarette smoke, which can augment proteolytic damage to matrix components and thus enhance the development of emphysema.

Increased oxidative stress in the airspaces can initiate a number of early inflammatory events in the lungs.

Oxidative Stress in the Airspaces

By virtue of its direct contact with the environment, the airspace epithelial surface of the lung is particularly vulnerable to the effects of oxidative stress. The respiratory tract lining fluid (RTLF) forms an interface between the epithelial cells and the external environment, and thus constitutes a first line of defense against inhaled oxidants.
At least three processes may be responsible for oxidant injury to the respiratory tract epithelial cells from cigarette smoke: (1) a direct toxic interaction of constituents of cigarette smoke (including free radicals) that have penetrated the protective antioxidant shield of the RTLF; (2) damage to the cells by toxic reactive products generated by interaction between cigarette smoke and RTLFs; and (3) reactions occurring subsequent to activation of inflammatory-immune processes initiated by (1) and/or (2), above.15–17

Injury to the epithelium may be an important early event following exposure to cigarette smoke, and is shown by an increase in airspace epithelial permeability.18 Lannan and colleagues19 demonstrated the injurious effect of both whole and vapor phases of cigarette smoke on human alveolar epithelial cell monolayers, as shown by increased epithelial cell detachment, decreased cell adherence, and increased cell lysis.

These effects were in part oxidant mediated, since they were partially prevented by the antioxidant glutathione (GSH) in concentrations (500 μM) that are present in the ELF. Extra- and intracellular GSH appears to be critical to the maintenance of epithelial integrity following exposure to cigarette smoke. This was shown in studies by Li et al.20,21 and Rahman et al.22 which demonstrate that the increased epithelial permeability of epithelial cell monolayers in vitro and in rat lungs in vivo following exposure to CSC was associated with profound changes in the homeostasis of the antioxidant GSH. Concentrations of GSH were considerably decreased, concomitant with a decrease in the activities of the enzymes involved in the GSH redox cycle such as GSH peroxidase (GP) and glucose-6-phosphate dehydrogenase. In addition, depletion of lung GSH alone, by treatment with the GSH-synthesis inhibitor buthionine sulfoximine, can induce increased airspace epithelial permeability both in vitro and in vivo.21–23

Similar results to these in vitro and animal studies were shown in human studies demonstrating increased epithelial permeability in chronic smokers compared with non-smokers, as measured by increased 99mTc-technetium-diethylenetriaminepentaacetic acid lung clearance, with a further increase in 99mTc-technetium-diethylenetriaminepentaacetic acid clearance following acute smoking.24 Thus, cigarette smoke has a detrimental effect on alveolar epithelial cell function that is, in part, oxidant mediated, since antioxidants provide protection against this injurious event.

The oxidant burden in lungs may be further enhanced in smokers by the increased numbers of neutrophils (by 10-fold) and macrophages (by two- to fourfold) in the alveolar space.25,26 In vitro studies have shown that the spontaneous release of ROS from alveolar leukocytes in cigarette smokers is increased, compared to those from nonsmokers.27–31 Recent evidence from bronchial biopsy and lung resection studies indicates that increased numbers of neutrophils present in both bronchial and alveolar walls in smokers with moderately severe COPD.32 Xanthine/xanthine oxidase, which generates O2•−, has also been shown to be increased in the BAL fluid (BALF) from patients with COPD.33

Oxidative Stress and Neutrophil Sequestration and Migration in the Lungs

The first step in the recruitment of neutrophils to the airspaces is the sequestration of these cells in the lung microcirculation.34 This occurs under normal circumstances in the pulmonary capillary bed, as a result of the size differential between neutrophils (average diameter, 7 μm) and pulmonary capillary segments (average diameter, 5 μm). Thus a proportion of the circulating neutrophils have to deform in order to negotiate the smaller capillary segments. Studies using a variety of techniques, including radiolabeled or fluorescent-labeled neutrophils, have supported the idea that the lungs contain a large pool of noncirculating neutrophils, which are either retained or slowly moving within the pulmonary microcirculation. In healthy subjects, radiolabeled neutrophil studies indicate that a proportion of neutrophils are normally delayed in the pulmonary circulation, compared to radiolabeled erythrocytes.35 In normal subjects, studies have shown a correlation between neutrophil deformability measured in vitro and the subsequent sequestration of these cells in the pulmonary microcirculation following their re-injection—the less deformable the cells, the increased sequestration of these cells occurs in the pulmonary circulation.35 This provides a mechanism for the creation of a pool of sequestered or noncirculating cells in the pulmonary microcirculation, without the need to invoke margination of neutrophils in the postcapillary venules, which is the mechanism for the noncirculating pool of cells in the systemic circulation.36 The sequestration of neutrophils in the pulmonary capillaries allows time for the neutrophils to interact with the pulmonary capillary endothelium, resulting in their adherence to the endothelium and thereafter their transmigration across the alveolar capillary membrane to the interstitium and airspaces of the lungs in response to inflammation or infection.

Any circumstances that lead to a decrease in neutrophil deformability will potentially increase neutrophil sequestration in the lungs.

Decreased neutrophil deformability occurs in cell activation due to the assembly of the cytoskeleton, in particular the polymerization of microfilaments (F actin), resulting in cell stiffening. Neutrophils can be activated while in transit in the pulmonary microcirculation by a number of mediators, including cytokines released from resident lung cells, alveolar macrophages, and epithelial and endothelial cells. Noxious inhaled agents, such as cigarette smoke, could influence the transit of cells in the pulmonary capillary bed. Studies in man using radiolabeled neutrophils and RBCs show a transient increase in neutrophil sequestration in the lungs during smoking, which returns to normal after cessation of smoking. Using an in vitro positive pressure cell filtration technique, it has been shown that cells exposed to cigarette smoke in vitro decrease their deformability.38 A similar decrease in deformability can be demonstrated in vivo for neutrophils in blood obtained from subjects who are actively smoking (Fig 1).39 Since each puff of cigarette smoke contains 1016 oxidant molecules, it has been suggested that the effect of cigarette smoke on neutrophil deformability is oxidant
mediated. Support for this hypothesis comes from in vitro studies that show that the decrease in neutrophil deformability induced by cigarette smoke exposure is abolished by antioxidants, such as GSH (Fig 2). There is also evidence that oxidative stress may reach the circulation during cigarette smoking, which could decrease the deformability of neutrophils, so increasing their sequestration in the pulmonary microcirculation. Oxidants appear to affect neutrophil deformability by altering the cytoskeleton by polymerizing actin.

Thus, cigarette smoking increases neutrophil sequestration in the pulmonary microcirculation, at least in part, by decreasing neutrophil deformability.

Once sequestered, components of cigarette smoke can alter neutrophil adhesion to endothelium by upregulating CD18 integrins, which is known to upregulate the nicotinamide-adenine dinucleotide phosphate oxidase-hydrogen peroxide (NADPH) generating system. Cigarette smoke has also been shown to alter neutrophil adhesion. Inhalation of cigarette smoke by hamsters increases neutrophil adhesion to the endothelium of both arterioles and venules. This increased neutrophil adhesion is thought to be mediated by oxygen radicals derived from cigarette smoke, since it was inhibited by pretreatment with CuZnSOD. Neutrophils sequestered in the pulmonary circulation of the rabbit following cigarette smoke inhalation also show increased expression of CD18 integrins, which is known to upregulate the NADPH oxidase-O2- generating system.

Increased expression of adhesion molecules in smoke-exposed animals may result from the secondary inflammatory effects of smoking, through the release of cytokines, since direct smoke exposure in vitro does not produce increased expression of neutrophil adhesion molecules, nor does it enhance functional adherence. Thus, several mechanisms involving oxidants cause neutrophil sequestration in the pulmonary microcirculation in smokers. Oxidant-mediated mechanisms may also result in the increased sequestration of neutrophils, which occurs in the microcirculation during exacerbations of COPD.

These sequestered neutrophils may subsequently respond to chemotactic components in cigarette smoke and become more adhesive to pulmonary vascular endothelial cells, in preparation for migration into the airspaces. Studies in animal models of smoke exposure have demonstrated increased neutrophil sequestration in the pulmonary microcirculation in situ, associated with upregulation of adhesion molecules on the surface of these cells. Activation of neutrophils sequestered in the pulmonary microvasculature could also induce the release of reactive oxygen intermediates and proteases within a microenvironment, with limited access for free radical scavengers and antiproteases. Thus, destruction of the alveolar wall, as occurs in emphysema, could result from a proteolytic insult derived from the intravascular space, without the need for the neutrophils to migrate into the airspaces.

As indicated above, several studies have shown that there are increased numbers of neutrophils in the BAL in chronic cigarette smokers. Neutrophil sequestration in the microcirculation allows chemotaxis to occur. Smoke exposure in humans results in increased chemotactic activity or levels of chemotactic factors in the airspaces.

**Evidence of Oxidative Stress in Smokers and Patients With COPD**

There is now overwhelming evidence for the presence of increased oxidative stress in smokers and patients with COPD. Increased oxidative stress mediates the increased expression of adhesion molecules in smokers and patients with COPD.

![Figure 1](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21946/ on 04/02/2017)

![Figure 2](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21946/ on 04/02/2017)
COPD. Direct measurements of specific markers of oxidative injury resulting from excessive free radical activity can be made by electron spin resonance, which cannot be applied to the study of tissues at present. Most studies have therefore relied on indirect measurements of free radical activity in biological fluids. Although these markers suggest that oxidative stress has occurred, they do not indicate that this event is necessarily involved in the pathogenesis of the condition that is being studied. Markers of oxidative stress have been shown to occur in the ELF, in the breath, and in the urine in cigarette smokers and patients with COPD.

Oxidative Stress and Proteinase/Antiproteinase Imbalance

The concept that a proteinase/antiproteinase imbalance occurs in the lungs as part of the pathogenesis of emphysema in smokers developed from studies of $\alpha_1$-antitrypsin ($\alpha_1$-AT)-deficient patients. In the case of smokers with normal levels of $\alpha_1$-AT, the elastase burden may be increased as a result of increased recruitment of leukocytes to the lungs, and there may be a functional deficiency of $\alpha_1$-AT due to inactivation in the lungs by oxidation, which is considered to contribute to the pathogenesis of this condition.

A large body of literature has been published in an attempt to prove the protease/antiprotease theory of the pathogenesis of emphysema. It is clear that an imbalance between an increased elastase burden in the lungs and a functional deficiency of $\alpha_1$-AT due to its inactivation by oxidants is an oversimplification, not least because other proteinases and other antiproteinases are likely to have a role. Early studies showed that the function of $\alpha_1$-AT in BAL was reduced by around 40% in smokers, compared with nonsmokers. This functional $\alpha_1$-AT deficiency is thought to be due to inactivation of the $\alpha_1$-AT by oxidation of the methionine residue at its active site by oxidants in cigarette smoke, as part of the pathogenesis of emphysema. Another major inhibitor of neutrophil elastase is secretory leukoprotease, which can also be inactivated by oxidants.

This theory was supported by in vitro studies showing loss of $\alpha_1$-AT inhibitory function when treated with oxidants, including cigarette smoke. In addition, oxidation of the methionine residue in $\alpha_1$-AT was confirmed in the lungs of healthy smokers. These studies supported the concept of inactivation of $\alpha_1$-AT by oxidation of the active site of the protein. Other studies showed that macrophages from the lungs of smokers release increased amounts of ROS, which could also inactivate $\alpha_1$-AT in vitro. However, most of the $\alpha_1$-AT in cigarette smokers remains active, and is therefore still capable of protecting against the increased protease burden. Later studies have provided conflicting data on whether $\alpha_1$-AT function lavage is altered in cigarette smokers, which may be due to technical differences between the studies that may have affected $\alpha_1$-AT function.

The acute effects of cigarette smoking on the functional activity of $\alpha_1$-AT in BALF have shown a transient, but nonsignificant fall in the antiprotease activity of BALF 1 h after smoking. Thus studies assessing the function of $\alpha_1$-AT in either chronic or acute cigarette smoking have failed to produce a clear picture.

Antioxidants in BALF

The major antioxidants in RTLF include mucin, reduced GSH, uric acid, protein (largely albumin), and ascorbic acid. Mucin is a glycoprotein rich in cysteine residues (sulphydrys), and hence is an important antioxidant of the RTLF. Mucins have metal binding properties that effectively scavenge hydroxyl radicals and would be expected to scavenge OCL/ HOCL because of the abundance of sulphydryl and disulphide moieties in their structure. Toxic inhalants increase the secretion of mucins, which therefore represent a major antioxidant in the upper RTLFs. However, oxygen radicals are known to degrade mucous glycoproteins. It is therefore likely that cigarette smoke oxidants also react with this respiratory tract secretory glycoprotein.

There is limited information on the respiratory epithelial antioxidant defenses in smokers, and less in COPD. Several studies have shown that GSH is elevated in BALF in the airways of chronic smokers. Despite the twofold increase in BALF GSH in chronic smokers, GSH may not be present in sufficient quantities to deal with the excessive oxidant burden during acute smoking when acute depletion of GSH may occur. Rahman and colleagues studied the acute effects of CSC on GSH metabolism in a human alveolar epithelial cell line in vitro, and in vivo in rat lungs after intratracheal CSC instillation. They found a dose and time-dependent depletion of intracellular GSH, concomitant with the formation of GSH conjugates, which is supported by similar results in studies in animal lungs in vivo. Furthermore, the activities of GSH redox system enzymes, such as GP and glucose 6-phosphate dehydrogenase, were transiently decreased in alveolar epithelial cells and in rat lungs after CSC exposure, possibly as a result of the action of highly electrophilic free radicals on the active site of the enzymes. GSH homeostasis may also play a central role in the maintenance of lung airspace epithelial barrier integrity. In particular, lowering the levels of GSH in epithelial cells leads to loss of barrier function and increased permeability.

Pacht and coworkers showed reduced levels of vitamin E in the BALF of smokers compared with nonsmokers. By contrast, Bui and colleagues found a marginal increase in vitamin C in BALF of smokers, compared to nonsmokers. Similarly, alveolar macrophages from smokers have both increased levels of ascorbic acid and augmented uptake of ascorbate. Enhanced activity of antioxidant enzymes (superoxide dismutase [SOD], and catalase) in alveolar macrophages from young smokers has also been reported. However, Kondo and coworkers found that increased superoxide generation by alveolar macrophages in elderly smokers was associated with decreased antioxidant enzyme activities, when compared with nonsmokers. The activities of CuZnSOD, GSH-S-transferase, and GP were found to be decreased in alveolar macrophages from elderly smokers. This reduced activity was
Evidence of Systemic Oxidative Stress

There has recently been considerable interest in the systemic effects of COPD. One manifestation of a systemic effect is the presence of markers of oxidative stress in the blood in patients with COPD. This is reflected in the increased sequestration of neutrophils in the pulmonary microcirculation during smoking and during exacerbations of COPD which, as described above, is an oxidant-mediated event.37–40,46

Rahman and colleagues40 demonstrated increased production of superoxide anion from peripheral blood neutrophils obtained from patients with acute exacerbations of COPD, which returned to normal when the patients were restudied when clinically stable. Other studies have shown that circulating neutrophils from patients with COPD have upregulated surface adhesion molecules, which may also be an oxidant-mediated effect.40,41 Neutrophil activation may be even more pronounced in neutrophils that are sequestered in the pulmonary microcirculation in smokers and in patients with COPD, since animal models of lung inflammation have shown that neutrophils which are sequestered in the pulmonary microcirculation release more ROS than circulating neutrophils in the same animal.40 Thus, neutrophils which are sequestered in the pulmonary microcirculation may be a source of oxidative stress, which may have a role in inducing airway injury in COPD, particularly during exacerbations.

Polysaturated fats and fatty acids in cell membranes are a major target for free radical attack, resulting in lipid peroxidation, a process that may continue as a chain reaction to generate peroxides and aldehydes. Products of lipid peroxidation reactions can be measured in body fluids as thiobarbituric acid reactive substances (TBARS). The levels of TBARS in plasma or in BALF, are significantly increased in healthy smokers and patients with acute exacerbations of COPD, compared with healthy nonsmokers.9–40 There is, however, a problem with the specificity of thiobarbituric acid-malondialdehyde assays as a measure of lipid peroxidation, since this assay does not directly measure the lipid peroxidation reaction. Other studies have measured conjugated levels of dienes of linoleic acid, a secondary product of lipid peroxidation, and shown the levels in plasma were elevated in chronic smokers.82 In addition, circulating levels of F 2-isoprostane, which is a more direct measurement of lipid peroxidation, have been found in smokers.83 Similarly Lapenna and colleagues84 demonstrated increased levels of fluorescent products of lipid peroxidation in smokers.

A recent study directly examined the balance between oxidants/antioxidants in smokers and patients with acute exacerbations of COPD by measuring changes in the antioxidant capacity in the blood. Rahman and coworkers40 found that the plasma antioxidant capacity was significantly decreased in smokers 1 h after smoking and in patients with acute exacerbations of COPD, when compared with plasma from age-matched nonsmoking control subjects (Fig 3). The decrease in plasma antioxidant capacity in smokers may be due to a profound depletion of plasma protein sulfydryls as demonstrated following cigarette smoke exposure in vitro.85–88 Thus, there is clear evidence that oxidants in cigarette smoke, either in vitro or in vivo, markedly decrease low molecular plasma antioxidants both in vitro and in vivo. Depletion of plasma antioxidants reduces the protection against cigarette smoke-induced plasma membrane peroxidation.

It is possible that individual variations in the ability to enhance the antioxidant screen in body fluids may be one factor that accounts for the susceptibility of some smokers to develop COPD.

Likewise, investigators have measured the major plasma antioxidants in smokers.90–95 These studies show a depletion of ascorbic acid vitamin E, beta-carotene, and sele-
nium in the serum of chronic smokers. Moreover, decreased vitamin E and vitamin C levels were measured in leukocytes from smokers. However, circulating RBCs from cigarette smokers contain increased levels of SOD and catalase, despite similar activity of GP, and are better able to protect endothelial cells from the effects of hydrogen peroxide, when compared with cells from nonsmokers.

Plasma ascorbate may be a particularly important antioxidant in the plasma because the gas phase of cigarette smoke induces lipid peroxidation in plasma in vitro that is decreased by ascorbate. Inhalation of NO from cigarette smoke, as well as NO and O$_2^-$ released by activated phagocytes react to form peroxynitrite, which is cytotoxic. Peroxynitrite has recently been shown to decrease plasma antioxidant capacity by rapid oxidation of ascorbic acid, uric acid, and plasma sulfhydryls. Evidence of NO/peroxynitrite activity in plasma has been demonstrated in cigarette smokers. Nitrification of tyrosine residues or proteins in plasma leads to the production of 3-nitrotyrosine. Petruzzelli and colleagues demonstrated the presence of 3-nitrotyrosine in plasma in smokers, which were possibly in higher levels than in a small group of nonsmokers. They also confirmed low levels of antioxidant capacity in smokers, which were negatively correlated with the levels of 3-nitrotyrosine. The levels of antioxidant capacity in the plasma have a negative correlation with the increased release of oxygen radicals from circulating neutrophils in patients with exacerbations of COPD.

OTHER MECHANISMS RELATED TO THE PATHOGENESIS OF COPD INVOLVING OXIDANTS

The majority of the information that is available on the pathogenesis of COPD relates to the development of emphysema. COPD also includes the other conditions of chronic bronchitis and small airways disease. It is presumed that the factors that initiate inflammation and the effects of proteolytic and oxidant-induced damage are also relevant to these conditions, although much less information is available.

Animal models of elastase-induced emphysema also show features of airways diseases with goblet cell hyperplasia. Neutrophil elastase is known to be a potent secretagogue for mucus glands, and therefore may contribute to the hypermucous secretion in chronic bronchitis. Oxidant-generated systems, such as xanthine/xanthine oxidase have also been shown to cause the release of mucus.

EVIDENCE FOR A RELATIONSHIP BETWEEN OXIDANT/ANTIOXIDANT BALANCE AND THE DEVELOPMENT OF AIRWAYS OBSTRUCTION

The neutrophil appears to be a critical cell in the pathogenesis of COPD. Previous epidemiologic studies have shown a relationship between circulating neutrophil numbers and the FEV$_1$. Indeed a relationship has been shown between the change in peripheral blood neutrophil count and the change in airflow limitation over time. Other studies have provided supportive evidence of a role for ROS released from circulating neutrophils and the development of airflow limitation. Richards and colleagues have shown a relationship between peripheral blood neutrophil chemiluminescence and measures of airflow limitation in young cigarette smokers. Even passive cigarette smoking has been associated with increased peripheral blood leukocyte counts and enhanced release of oxygen radicals. Oxidative stress, measured as TBARS in plasma, has also been shown to correlate inversely with the percent predicted FEV$_1$ in a population study, indicating that lipid peroxidation is associated with airflow limitation in the general population.

An association between dietary intake of antioxidant vitamins and lung function has been demonstrated in the general population. Britton and coworkers showed in a population of 2,633 subjects an association between dietary intake of the antioxidant vitamin E and lung function, supporting the hypothesis that this antioxidant may have a role in protecting against the development of COPD; hence, vitamin supplementation may be a possible preventive therapy against the development of COPD. Such intervention studies have been difficult to carry out, but there is at least some evidence to suggest that antioxidant vitamin supplementation reduces oxidant stress, measured as a decrease in pentane levels in breath as an assessment of lipid peroxides.

OXIDATIVE STRESS AND GENE EXPRESSION

Proinflammatory Genes

There is overwhelming evidence that COPD is associated with airway and airspace inflammation, as shown for example by recent biopsy studies. Numerous markers of inflammation have been shown to be elevated in the sputum of patients with COPD, such as interleukin (IL)-8 and tumor necrosis factor (TNF)-α.

Genes for many inflammatory mediators, such as the cytokines IL-8, TNF-α, and nitric oxide (NO) are regulated by transcription factors such as NF-κB. NF-κB is present in the cytosol in an inactive form linked to its inhibitory protein IκB. Many stimuli, including cytokines and oxidants, activate NF-κB, resulting in ubiquination cleaving of IκB from NF-κB and the destruction of IκB in the proteosome. This critical event in the inflammatory response is redox sensitive. We have shown in preliminary studies in vitro, using both macrophage cell lines and alveolar and bronchial epithelial cells, that oxidants cause the release of inflammatory mediators such as IL-8, IL-1, and NO, and that these events are associated with increased expression of the genes for these inflammatory mediators and increased nuclear binding or activation of NF-κB.

Thiol antioxidants such as N-acetylcysteine (NAC) and nacystein, which have potential as therapies in COPD, have been shown in in vitro experiments to block the release of these inflammatory mediators from epithelial cells and macrophages, by a mechanism involving increasing intracellular GSH and decreasing NF-κB activation.
**Antioxidant Genes**

As described above, there is considerable evidence for an increased oxidant burden in the lungs of smokers and patients with COPD. An important effect of oxidative stress is the upregulation of protective antioxidant genes. The antioxidant GSH is concentrated in ELF compared with plasma,\(^{115}\) and appears to have an important protective role, together with its redox enzymes in the airspaces and intracellularly in epithelial cells. To illustrate the protective role of GSH against the effects of cigarette smoke, we have developed models \textit{in vitro} in the rat and \textit{in vivo} using monolayer cultures of alveolar epithelial cells, to assess the injurious effects of cigarette smoke. Human studies have shown that GSH is elevated in ELF in chronic cigarette smokers, compared with nonsmokers,\(^{65}\) an increase that does not occur during acute cigarette smoking.\(^9\) The effects of acute and chronic cigarette smoking can be mimicked following intratracheal instillation of CSC in the rat and exposure of epithelial cell monolayers to cigarette smoke \textit{in vitro}.\(^{20,21,68}\) Following exposure to cigarette smoke, there is a profound decrease in GSH in BAL in the rat that is mirrored by a fall in total lung GSH 6 h after exposure.\(^{21,68}\) Similarly, there is a fall in intracellular GSH in epithelial cells following exposure to CSC.\(^{20,68}\) There is an association between the fall in lung and intracellular GSH both \textit{in vivo} and \textit{in vitro} and the increase in epithelial permeability, as described above.

We have used a rat model of intratracheal instillation of CSC \textit{in vivo} and exposure of epithelial cell monolayers \textit{in vitro} to study the regulation of GSH and its redox system in response to CSC and other oxidents, and in particular to investigate the discrepancy between GSH levels in chronic and acute cigarette smoking. After exposure of airspace epithelial to CSC \textit{in vitro}, there is an initial decrease in intracellular GSH with a rebound increase when the cells are washed and culture is continued for 24 h.\(^{116}\) This effect \textit{in vitro} was mimicked by a similar change in GSH in rat lungs \textit{in vivo} following intratracheal instillation of CSC,\(^{68}\) associated with an increase in oxidized GSH. We also examined the activity of the major enzymes involved in GSH synthesis and in the GSH redox system in response to CSC both \textit{in vivo} and \textit{in vitro}. The initial fall in lung and intracellular GSH after treatment with CSC was associated with a decrease in the activity of \textit{γ}-glutamylcysteine synthetase (\textit{γ}-GCS), the rate-limiting enzyme of GSH synthesis, with recovery of the activity by 24 h.\(^{98,115}\) We hypothesize that the increased levels of GSH following CSC exposure may be due to induction of the \textit{γ}-GCS gene by components within cigarette smoke. Using reverse transcriptase-polymerase chain reaction, we showed an increase in \textit{γ}-GCS messenger RNA expression 12 to 24 h after airspace epithelial cells were exposed to CSC \textit{in vitro} (Fig 4).\(^{116,117}\) We also demonstrated that the upregulation of \textit{γ}-GCS gene expression occurred at the transcriptional level (modified from Rahman et al\(^{116}\)).

![](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21946/ on 04/02/2017)
GSH as a result of AP-1 activation and an increased \( \gamma \)-GCS expression.\(^{119} \)
Corticosteroids have been used as anti-inflammatory agents in COPD, but there is still doubt over their effectiveness in reducing airway inflammation in COPD. Interestingly dexamethasone also causes a decrease in intracellular GSH in airspace epithelial cells, but no rebound increase compared with the effects of TNF.\(^{119} \)

Moreover, the rebound increase in GSH produced by TNF in epithelial cells is prevented by cotreatment with dexamethasone.\(^{119} \) These effects may have relevance for the treatment of COPD patients with corticosteroids.

Recently, Gilks and coworkers\(^{120} \) have shown in rats exposed to whole cigarette smoke for up to 14 days an increase in the expression of a number of antioxidant genes in their bronchial epithelial cells. Whereas the expression messenger RNA of manganese SOD and metallothionein was increased at 1 to 2 days and returned to normal by 7 days, messenger RNA for GP did not increased until 7 days exposure, suggesting the importance of the GSH redox system as a mechanism for chronic protection against the effects of cigarette smoke.

The \( cfos \) gene belongs to a family of growth- and differentiation-related immediate early genes, the expression of which generally represents the first measurable response to a variety of chemical and physical stimuli.\(^{112} \) Studies in various cell lines have shown enhanced gene expression of \( cfos \) in response to CSC.\(^{121} \) These effects of CSC can be mimicked by peroxynitrite and smoke-related aldehydes in concentrations that are present in CSC.\(^{121} \) The effects of CSC can be enhanced by pretreatment of the cells with buthionine sulfoxamine to decrease intracellular GSH and can be prevented by treatment with NAC, a thiol antioxidant.\(^{121} \) These studies emphasize the importance of the intracellular levels of the antioxidant GSH in gene expression.

Thus oxidative stress, including that produced by cigarette smoke, causes increased gene expression of both proinflammatory genes by oxidant-mediated activation of...
transcription factors such as NF-κB, and activation of protective genes such as γ-GCS through other transcription factors, which in the case of γ-GCS, is the transcription factor AP-1. A balance may therefore exist between pro- and anti-inflammatory gene expression in response to cigarette smoke, which may be critical to whether cell injury is induced by cigarette smoking (Fig 6). Knowledge of the molecular mechanisms that regulate these events may open new therapeutic avenues in the treatment of COPD.

**Oxidative Stress and Susceptibility to COPD**

Since only a proportion (15 to 20%) of cigarette smokers appear to be susceptible to its effects and show a rapid decline in FEV₁ and develop the disease,¹² there has been considerable interest in identifying those who are most susceptible and the mechanisms of that susceptibility,¹²³ since this may provide an important insight into the pathogenesis of COPD, as did the recognition of an association between α₁-AT and COPD.

Polymorphisms of various genes have been shown to be more prevalent in smokers who develop COPD than in nonsmokers.¹²¹ A number of these polymorphisms may have functional significance, such as the association between the TNF-α gene polymorphism (TNF-2) which may be associated with increased TNF levels in response to inflammation, and the development of chronic bronchi-

tis.¹²⁴ Relevant to the effects of cigarette smoke is a polymorphism in the gene for microsomal epoxide hydrolase, which is an enzyme involved in the metabolism of highly reactive epoxide intermediates that are present in cigarette smoke.¹²⁵ The proportion of individuals with a slow microsomal epoxide hydrolase activity (homozygotes) was significantly higher in patients with COPD and a subgroup of patients shown pathologically to have emphysema (COPD, 22%; emphysema, 19%) compared with control subjects (6%). It may be that a panel of the susceptibility polymorphisms of functional significance in enzymes involved in xenobiotic metabolism or antioxidant enzyme genes may allow individuals to be identified as being susceptible to the effects of cigarette smoke.

**Therapeutic Options to Redress the Oxidant/Antioxidant Imbalance in COPD**

Having demonstrated evidence for an oxidant/antioxidant imbalance in smokers and its probable role in the pathogenesis of COPD, do we have any therapeutic options? Various approaches have been tried to redress this imbalance. One approach would be to target the inflammatory response by reducing the sequestration or migration of leukocytes from the pulmonary circulation into the airspaces. Possible therapeutic options for this are drugs that alter cell deformability, so preventing neutrophil sequestration or the migration of neutrophils, either by interfering with adhesion molecules necessary for migra-
tion, or preventing the release of inflammatory cytokines such as IL-8 or leukotriene-B4, which result in neutrophil migration. It should also be possible to use anti-inflammatory agents to prevent the release of oxygen radicals from activated leukocytes or to quench those oxidants once they are formed, by enhancing the antioxidant screen in the lungs.

There are various options to enhance the lung antioxidant screen. One approach would be the molecular manipulation of antioxidant genes, such as GP or genes involved in the synthesis of GSH, such as γ-GCS or by developing molecules with activity similar to those of antioxidants enzymes such as catalase and SOD.

Another approach would simply be to administer antioxidant therapy. This has been attempted in cigarette smokers using various antioxidants such as vitamin C and vitamin E. Attempts to supplement lung GSH have been tried using GSH or its precursors. Nebulized GSH has also been used therapeutically, but this has been shown to induce bronchial hyperreactivity. Cysteine is a thiol that is the rate-limiting amino acid in GSH synthesis. Cysteine administration is not possible since it is oxidized to cysteine that is neurotoxic. The cysteine-donating compound NAC acts as a cellular precursor of GSH and becomes de-acetylated in the gut to cysteine following oral administration. It reduces disulphide bonds and has the potential to interact directly with oxidants. The use of NAC in an attempt to enhance GSH in patients with COPD has met with varying success. NAC given orally in low dosages, 600 mg/d, to normal subjects results in very low levels of NAC in the plasma for up to 2 h after administration. Bridgeman and colleagues showed after 5 days of NAC, 600 mg tid, that there was a significant increase in plasma GSH levels. However, there was no associated rise in BAL GSH or in lung tissue. These data seem to imply that producing a sustained increase in lung GSH is difficult using NAC in subjects who are not already depleted of GSH. In spite of this, continental European studies have shown that NAC reduces the number of exacerbation days in patients with COPD. This was not confirmed in a British Thoracic Society study of NAC. The contradictory results of these studies may result from several reasons. Firstly, the positive studies of NAC were in patients who had relatively mild COPD, whereas in the British study the patients had more severe COPD. Secondly, a relatively small dose of NAC was given in both studies.

N-acystelyn (NAL) is a lysine salt of N-acetylinecysteine. It is also a mucolytic and oxidant thiol compound that, in contrast to NAC, which is acid, has a neutral pH. NAL can be aerosolized into the lung without causing significant side effects. Studies comparing the effects of NAL and NAC found that both drugs enhanced intracellular GSH in alveolar epithelial cells and inhibited hydrogen peroxide and superoxide anion release from neutrophils harvested from peripheral blood from smokers and patients with COPD.

Most animal cells normally export GSH, and do not take up intact GSH. GSH ethyl ester contains an ethyl group that is esterified to the glycine of GSH. GSH ethyl ester is more lipophylic and thus passes more readily into cells than GSH. The monoester is then hydrolyzed to GSH by cytosolic nonspecific esterase. GSH monoethyl ester is resistant to the cleavage by the enzyme γ-glutamylcysteine transpeptidase and has been used to increase GSH in vitro. Thiazolidine is a potentially useful compound for cysteine delivery and can be shown to protect against oxidative injury. However, there are no studies in humans that validate these compounds for clinical trials.

Molecular regulation of GSH synthesis by targeting...
\( \gamma \)-GCS has great promise in as a means of treating oxidant medicated injury in the lungs. Cellular GSH may be increased by increasing \( \gamma \)-GCS activity. This may be possible by gene transfer techniques, although this would be an expensive treatment that may not be considered for a condition such as COPD. However, knowledge of how \( \gamma \)-GCS is regulated may allow the development of other compounds that may act to enhance GSH.

In summary, there is now very good evidence for an oxidant/antioxidant imbalance in COPD and increasing evidence that this imbalance is important in the pathogenesis of this condition. There are a number of important effects of oxidative stress in smokers that are relevant to the development of COPD (Fig 7). Oxidative stress may also be critical to the inflammatory response to cigarette smoke, through the upregulation of redox-sensitive transcription factors and hence proinflammatory gene expression; but it is also involved in the protective mechanisms against the effects of cigarette smoke by the induction of antioxidant genes. Inflammation itself induces oxidative stress in the lungs, and polymorphisms on genes for inflammatory mediators or antioxidant genes may have a role in the susceptibility to the effects of cigarette smoke. Knowledge of the mechanisms of the effects of oxidative stress should in the future allow the development of potent antioxidant therapies that test the hypothesis that oxidative stress is involved in the pathogenesis of COPD, not only by direct injury to cells, but also as a fundamental factor in inflammation in smoking-related lung disease.

REFERENCES

34 MacNee W, Selby C. Neutrophil traffic in the lungs, the role of hemodynamics, cell adhesion and deformability. Thorax 1993; 48:79–88
64 Cooper B, Creeth JM, Donald ASR. Studies of the limited degradation of mucus glycoproteins: the mechanism of the peroxide reaction. Biochem J 1985; 228:615–626
72 McCusker K, Hoidal J. Selective increase of antioxidant enzyme activity in the alveolar macrophages from cigarette smokers and smoke-exposed hamsters. Am Rev Respir Dis 1990; 141:678–682
73 Kondo T, Tagami S, Yoshioka A, et al. Current smoking of
89 Steinberg FM, Chait A, Antioksidant vitamin supplementation and lipid peroxidation in smokers Am J Clin Nutr 1998; 59:201S–205S
92 Watchorn T, Muller B, MacNee W. Does increasing intracellular glutathione inhibit cytokine-induced nitric oxide release and Nf-κB activation. Am J Respir Crit Care Med 1998; 157:A889
114 Parmentier M, Drozd E, Hirani N, et al. Thiol antioxidants inhibit neutrophil chemotaxis by decreasing release of IL-8 from macrophages and pulmonary epithelial cells. Am J Respir Crit Care Med 1999; 159:A286


121 Muller T, Gebel S. The cellular stress response induced by aqueous extracts of cigarette smoke is critically dependent on the intracellular glutathione concentration. Carcinogenesis 1998; 19:797–801


125 Smith CAD, Harrison DJ. Association between polymorphism in gene for microsomal epoxide hydrolase and susceptibility to emphysema. Lancet 1997; 350:630–633


144 Tsan MF, Phillips PG. L-2-oxothiazolidine-4-carboxylate protects cultured endothelial cells against hyperoxia-induced injury. Inflammation 12:113–121

**Neutrophil Elastase Induces MUC5AC Messenger RNA Expression by an Oxidant-Dependent Mechanism**

Bernard Fischer, DVM, PhD; and Judith Vognone, MD

(CHEST 2000; 117:317S–320S)

Airway diseases such as cystic fibrosis, chronic bronchitis, and viral- or pollution-triggered asthma have two common pathologic features: mucous obstruction of the airways, and neutrophil-predominant airway inflammation. Neutrophils release high concentrations of elastase (neutrophil elastase [NE]), a serine protease, into the airways; exposure to elastase results in secretory metaplasia and increased production/secretion of mucin glycoproteins. We have pre-

*From the Division of Pediatric Pulmonary Diseases, Duke University Medical Center, Durham, NC. Supported by the Cystic Fibrosis Foundation, The North Carolina Biotechnology Center, and Duke University Medical Center. Correspondence to: Judith A. Vognone, MD, Division of Pediatric Pulmonary Diseases, Duke University Medical Center, Box 2994, Durham, NC 27710; e-mail: vognone001@mc.duke.edu*