Treatment of stable COPD: antioxidants

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ABSTRACT: There is considerable evidence that an increased oxidative burden occurs in the lungs of patients with chronic obstructive pulmonary disease (COPD) and this results in an imbalance between oxidants/antioxidants or oxidative stress, which may play a role in many of the processes involved in the pathogenesis of COPD. These include enhanced proteolytic activity, mucus hypersecretion and the enhanced inflammatory response in the lungs to inhaling tobacco smoke, which is characteristic of COPD. COPD is now recognised to have multiple systemic consequences, such as weight loss and skeletal muscle dysfunction. It is now thought that oxidative stress may extend beyond the lungs and is involved in these systemic effects.

Antioxidant therapy therefore would seem to be a logical therapeutic approach in chronic obstructive pulmonary disease. There is a need for more potent antioxidant therapies to test the hypothesis that antioxidant drugs may be a new therapeutic strategy for the prevention and treatment of chronic obstructive pulmonary disease.

KEYWORDS: Antioxidants, chronic obstructive pulmonary disease, oxidative stress

The lungs are continuously exposed to oxidants generated either endogenously (e.g. released from phagocytes or intracellular oxidants, e.g. from mitochondrial electron transport) or exogenously (e.g. air pollutants or cigarette smoke). The lungs are protected against this oxidative challenge by well-developed enzymatic and nonenzymatic antioxidant systems. Oxidative stress is said to occur when the balance between oxidants and antioxidants shifts in favour of oxidants, from either an excess of oxidants and/or depletion of antioxidants (fig. 1) [1].

Smoking is the main aetiological factor in chronic obstructive pulmonary disease (COPD). Cigarette smoke contains 10^17 oxidant molecules per puff, and this, together with a large body of evidence demonstrating increased oxidative stress in smokers and in patients with COPD, has led to the proposal that an oxidant/antioxidant imbalance is important in the pathogenesis of this condition [2]. Oxidative stress not only produces direct injurious effects in the lungs, but also activates the molecular mechanisms that initiate lung inflammation [3] and may have a role in many of the processes thought to be involved in the complex pathological events that result in COPD.

Increasingly, COPD is recognised not only to affect the lungs, but to have significant systemic consequences, such as muscle dysfunction and weight loss [4]. Oxidative stress is also thought to play an important role in this aspect of the disease [5].

EVIDENCE OF OXIDATIVE STRESS IN THE LUNGS

Oxidative stress can be measured in several different ways, either as direct measurements of the oxidative burden, as the responses to oxidative stress or the effect of oxidative stress on target molecules. Numerous studies using various techniques have now shown that oxidative stress is increased in the lungs of COPD patients compared with healthy subjects, but also compared with smokers with similar smoking history, but who have not developed COPD [2].

INCREASED OXIDANT BURDEN IN COPD

Reactive oxygen (ROS) and reactive nitrogen species, in addition to other molecules, such as protein radicals and lipid peroxidation products, contribute to the oxidant/antioxidant imbalance seen in COPD. ROS are produced by the sequential reduction of oxygen. The addition of one electron to oxygen produces super oxide anion (O_2^-), the addition of a second electron results in hydrogen peroxide (H_2O_2) and a third yields the formation of hydroxyl radical (OH^-). These intermediates are called ROS and react readily with molecules such as proteins, lipids and DNA (fig. 2). The addition of a fourth electron produces the complete reduction of O_2^- to water, which no longer reacts with other molecules. A free radical is a molecule with at least one unpaired electron, such as the superoxide hydroxyl radical and nitric oxide (NO).

Cigarette smoke contains >5,000 different chemicals of which many are oxidants, including...
cigarette smoke and in the epithelial lining fluid and may catalyse the production of free radicals through the Fenton and Haber-Weiss reactions. Air pollutants are another exogenous source of reactive oxygen species in the lungs [8]. The lung is exposed to oxidants from the environment; however, a large proportion of oxidants produced endogenously in the lungs are by-products of normal cellular metabolism. Mitochondria are the largest source of free radicals, as a result of electron leak from the electron transport chain onto oxygen to form superoxide [7]. Xanthine/xanthine oxidase is another significant cytosolic source of superoxide [9]. Membrane oxidases, such as cytochrome P450 enzymes [10] and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, also produce substantial quantities of superoxide. Reactive nitrogen species are derived from NO, which is produced by the three NO synthases. NO is produced in high concentrations as a result of inducible NO activation and can react with oxygen or superoxide to form NO and peroxynitrite. NO can also react with thiols to produce nitrosothiols and can metabolise to nitrite.

**ANTIOXIDANTS IN THE LUNGS**

Antioxidants are usually classified as either enzymatic or nonenzymatic and are the primary defences against reactive oxygen/reactive nitrogen species (fig. 3). Antioxidant enzymes include the superoxide dismutase (SOD) family, catalase, glutathione (GSH) peroxidase, GSH S-transferase and thioredoxin [11].

The nonenzymatic antioxidants include low molecular weight compounds, such as GSH, ascorbate, urate, alpha-tocopherol, bilirubin and lipoic acid. Concentrations of these nonenzymatic antioxidants vary in the lungs. Some, for example GSH, are more concentrated in epithelial lining fluid compared with plasma [12] and others, such as albumin, are found in high concentration in serum, but at much lower concentrations in the epithelial lining fluid (table 1) [13].
There is now considerable evidence showing the presence of intracellular spaces [7]. with the blood [12, 25]. GSH is also highly concentrated in (BALF) contains 100-fold concentration of GSH compared through GSH S transferase. Bronchoalveolar lavage fluid via addition to detoxifying compounds oxidant. It is concentrated in the epithelial lining fluid and GSH is the most abundant intracellular thiol-based anti-
The glutathione redox system disulfide reduction.

The thioredoxin system
This system consists of multiple species, including the thioredoxins, thioredoxin-like proteins and the thioredoxin reductases [22–24]. These enzymes are important in protein disulfide reduction.

The glutathione redox system
GSH is the most abundant intracellular thiol-based antioxidant. It is concentrated in the epithelial lining fluid and plays a critical role in maintaining intracellular redox status, in addition to detoxifying compounds via conjugation reactions through GSH S transferase. Bronchoalveolar lavage fluid (BALF) contains 100-fold concentration of GSH compared with the blood [12, 25]. GSH is also highly concentrated in intracellular spaces [7].

OXIDATIVE STRESS IN COPD
There is now considerable evidence showing the presence of increased oxidative stress in COPD. Direct measurements of the airspace oxidative burden, such as measurements of H₂O₂ in exhaled breath condensate, thought to derive from increased release of O₂⁻ by alveolar macrophages [26], show higher levels in smokers and patients with COPD, than in ex-smokers with COPD, or nonsmokers [27, 28] (fig. 4). O₂⁻ and H₂O₂ are also derived from the xanthine/xanthine oxidase reaction, which shows increased activity in BALF and in the plasma of smokers and in patients with COPD, compared with healthy subjects and nonsmoking subjects [29, 30].

NO has been used as a marker of airway inflammation and indirectly as a measure of oxidative stress. Its rapid reactivity with O₂⁻ or with thiols may alter NO levels in breath and, as a result, increased levels of NO in exhaled breath occur in some [31–33], but not in other studies of patients with COPD [34, 35]. However, nitrosothiols have been shown to be higher in breath condensate in smokers and COPD patients, compared to nonsmoking subjects (fig. 5) [36]. In addition, peroxynitrite, formed by the reaction of NO with O₂⁻, can cause nitration of tyrosine to produce nitrotyrosine [37]. Nitrotyrosine levels are elevated in sputum leukocytes of COPD patients and are negatively correlated with forced expiratory volume in one second (FEV₁) [38].

Exhaled carbon monoxide (CO) as a measure of the response of Haem oxygenase to oxidative stress has been shown to be elevated in exhaled breath in CO compared with normal subjects [39].

Lipid peroxidation products, such as thiobarbituric acid-reacting substances and malondialdehyde, are elevated in sputum in patients with COPD and are negatively correlated with the FEV₁ [40–42].

Isoprostanes, produced by ROS-mediated peroxidation of arachidonic acid, are found in higher levels in breath condensate in patients with COPD, compared with normal subjects and smokers who have not developed the disease and have also been shown to correlate with the degree of airways obstruction [43]. The levels of 8-isoprostane in breath condensate in COPD patients also show a positive correlation with the percentage neutrophils in induced sputum, suggesting oxidative stress may be involved in the airway inflammation in COPD (fig. 6) [44]. Increased levels of isoprostanes in plasma in smokers [45] and in urine in COPD patients [46] reflect systemic oxidative stress.

### TABLE 1

| Antioxidant             | Plasma    | Epithelial lining fluid |
|-------------------------|-----------|-------------------------|
| Glutathione             | 1.0 ± 0.7 | 109 ± 64                |
| Urate                   | 378 ± 133 | 207 ± 167               |
| Ascorbate (Vitamin C)   | 67 ± 25   | 40 ± 18                 |
| α-tocopherol (Vitamin E)| 16 ± 5    | 0.7 ± 0.3               |

Data presented as mean ± so. in μM.

### FIGURE 4.
Hydrogen peroxide (H₂O₂) levels in exhaled breath condensate in clinically stable and unstable chronic obstructive pulmonary disease (COPD) patients. *: p<0.05; ***: p<0.001. Reproduced with permission [27].
Hydrocarbon gases are by-products of ROS peroxidation of fatty acids [39]. Increased levels of exhaled breath ethane occur in COPD patients compared with control subjects, and the levels are negatively correlated with lung function [47].

Increased markers of oxidative stress are also present in lung cells in COPD patients. Increased nitrotyrosine has been shown in sputum leukocytes in COPD and healthy subjects (fig. 5b) [38]. The lipid peroxidation product, 4-hydroxynonenal, reacts quickly with cellular proteins to form adducts, which are present in greater quantities in airway epithelial and endothelial cells in the lungs of COPD patients, compared with smokers with a similar smoking history who have not developed the disease (fig. 7) [48].

Thus there is considerable evidence of increased markers of oxidative stress in the airways and in lung tissue in COPD patients, compared with normal subjects and smokers who have not developed the disease. In addition, many of these markers of oxidative stress correlate with the degree of airflow limitation in COPD, suggesting a role for oxidative stress in the decline in lung function in COPD.

SYSTEMIC OXIDATIVE STRESS

An increased systemic oxidative burden has been shown to occur in smokers. In COPD peripheral blood neutrophils have been shown to release more reactive oxygen species than in normal subjects and this is enhanced still further in exacerbations of the disease, and is associated with marked depletion of the plasma antioxidant capacity, indicating increased systemic oxidative stress (fig. 8) [49]. Products of lipid peroxidation are also increased in plasma in smokers with COPD, particularly during exacerbations [49]. Increased levels of nitrotyrosine have also been shown to occur in the plasma of COPD patients [37].

Exacerbations of COPD are known to result from increased levels of air pollutants, specifically particulate air pollution [8]. Particulate air pollution causes oxidative stress in the airways [50] and enhanced inflammation by similar mechanisms to those described for cigarette smoke, through oxidative stress-induced transcription factor activation.

It is now recognised that COPD is not only a disease which affects the lungs, but has important systemic consequences, such as cahexia and skeletal muscle dysfunction [51]. Increasing evidence of similar mechanisms involving oxidative stress...
stress and inflammation in the lungs may also be responsible for many of the systemic effects of COPD [5, 52].

ANTIOXIDANTS IN COPD

A relationship between dietary antioxidants, pulmonary function and the development of COPD has been shown in several population studies. The American Nutrition Examination Survey (NHANES) and the Dutch monitoring project for the risk factors for chronic diseases (MORGAN) have shown relationships between antioxidant dietary intake and airflow limitation. In NHANES (I) lower dietary intake of vitamin C was related to lower FEV1 levels in a population survey [53]; data from NHANES (II) showed an inverse relationship between dietary and serum vitamin C and chronic respiratory symptoms [54] and in NHANES (III) the levels of dietary vitamin C, vitamin E, selenium and beta-carotene correlated positively with lung function [55]. Similarly the MORGAN study has also shown that a higher intake of vitamin C and beta-carotene was associated with higher levels of the FEV1. This and other studies have shown associations between fruit intake and higher FEV1 and lower symptoms in COPD patients [56–59].

As a response to oxidative stress induced by cigarette smoke, there appears to be upregulation of protective antioxidant genes in the lungs. The antioxidant GSH is elevated in the epithelial lining fluid in chronic cigarette smokers, compared with nonsmokers, an increase that does not occur during acute cigarette smoking [52]. The effects of chronic cigarette smoking can be mimicked by exposure of airspace epithelial cells to cigarette smoke condensate in vitro. This produces an initial decrease in intracellular GSH, with a rebound increase after 24 h [60, 61]. This effect in vitro is mimicked by a similar change in GSH in rat lungs in vivo following exposure to
cigarette smoke [61], associated with an increase in the oxidised form of GSH. The increase in GSH following cigarette smoke exposure is due to transcriptional upregulation of mRNA for γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis [61]. The mechanism of the upregulation of γ-glutamylcysteine synthetase mRNA is through the activation by cigarette smoke of the redox-sensitive transcription factor activator protein-1 (AP-1) [62, 63]. These events are likely to account for the increased levels seen in epithelial lining fluid in chronic cigarette smokers, which acts as a protective mechanism. The injurious effects of cigarette smoke may occur repeatedly during and immediately after cigarette smoking when the lung is depleted of antioxidants, including GSH. Animals exposed to whole cigarette smoke for up to 14 days also show increased expression of a number of antioxidant genes including manganese superoxide dismutase, metallothionein and GSH peroxidase [21].

OXIDATIVE STRESS AND THE PATHOGENESIS OF COPD

Although studies have shown that oxidative stress is increased in COPD and is related to the development of airflow limitation, this does not indicate cause and effect, since oxidative stress may be an epiphenomenon related to the presence of inflammation [64]. There are as yet no longitudinal studies showing that the presence of enhanced oxidative stress is related to the decline in FEV1, nor to the progression of the disease. However, there are many studies that indicate that oxidative stress is involved in most of the processes that are thought to be important in the pathogenesis of COPD.

A relative “deficiency” of antiproteases, such as alpha-1-antitrypsin, due to their inactivation by oxidants, is thought to create a protease-antiprotease imbalance in the lungs, which forms the basis of the protease/antiprotease theory of the pathogenesis of emphysema [65, 66]. Although inactivation of alpha-1-antitrypsin by oxidants from cigarette smoke or oxidants released from inflammatory leukocytes has been shown in vitro [67, 68], the effect is less apparent in vivo [69]. However, a protease/antiprotease imbalance involving alpha-1-antitrypsin and elastase is an oversimplification, since other proteases and other antiproteases are likely to have a role.

Oxidant generating systems, such as xanthine/xanthine oxidase, have been shown to cause the release of mucus from airway epithelium [70]. Oxidants are also involved in the signalling pathways for the epidermal growth factor receptor, which has an important role in the release of mucus [71]. Oxidants, such as H2O2 or hypochlorous acid, in low concentrations (100 μM) have been shown to cause significant impairment of ciliary beating and even complete ciliary stasis [72]. Thus, oxidant-mediated mucus hypersecretion and impaired mucociliary clearance may result in the accumulation of mucus in the airways contributing to airflow limitation.

Injury to the airway epithelium is an important early event following exposure to cigarette smoke, as shown by an increase in airspace epithelial permeability. Increased epithelial permeability can be shown to result from cigarette smoke exposure both in vitro and in vivo [52, 73, 74] and is partially reversible by antioxidants. Extra and intracellular GSH appears to be critical to the maintenance of epithelial integrity following exposure to cigarette smoke, since depletion of lung GSH alone can induce increased airspace epithelial permeability both in vitro and in vivo [74, 75]. Interindividual variability in antioxidant defences may be one factor in determining whether COPD develops in cigarette smokers.

There is now overwhelming evidence that COPD is associated with enhanced airway and airspace inflammation, as is shown in recent biopsy studies [76]. Oxidative stress may be a mechanism by which airspace inflammation is enhanced in COPD [77]. Oxidative stress may have a fundamental role enhancing inflammation through the upregulation of redox-sensitive transcription factors, such as nuclear factor-kB (NF-kB) and AP-1, and also through the extracellular signal-regulated kinase, jun-N-terminal kinase and p38 mitogen activated protein-kinase signalling pathways. Cigarette smoke has been shown to activate all of these signalling pathways [3, 78, 79]. Genes for many inflammatory mediators are regulated by oxidant sensitive transcription factors, such as NF-kB [80, 81]. In vitro studies, both in macrophage cell lines and, alveolar and bronchial epithelial cells, show that oxidants cause the release of inflammatory mediators, such as interleukin (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 and activation of NF-kB [82, 83]. NF-kB has been shown to be activated and translocated to the nucleus in lung tissue in smokers and in patients with COPD compared with healthy subjects [78, 84]. NF-kB activation in lung tissue has been shown to correlate with the FEV1 [85]. Linking of NF-kB to its consensus site in the nucleus leads to enhanced transcription of pro-inflammatory genes and hence inflammation, which itself will produce more oxidative stress, creating a vicious circle of enhanced inflammation resulting from the increased oxidative stress (fig. 9).

Animal models of smoke exposure show that neutrophil influx in the lungs is associated with increased IL-8 gene expression and protein release and with NF-kB activation [86, 87]. All of these events are associated with oxidative stress since they can be abrogated by antioxidant therapy [86].
A further event induced by oxidative stress that may enhance the inflammation in the lungs is chromatin remodelling, which allows access for transcription factors, such as NF-κB and RNA polymerase to the transcriptional machinery, so enhancing gene expression. This process is known to be oxidant sensitive [77].

A recent hypothesis in the pathogenesis of emphysema is that loss of lung cells in alveolar walls, specifically endothelial cells, may occur as an initial event in the development of emphysema as a result of endothelial cell apoptosis [88]. Apoptosis has been shown to occur to a greater extent in emphysematous lungs than in nonsmokers’ lungs [89]. The process of endothelial apoptosis is thought to be under the influence of the vascular endothelial growth factor receptor 2 (VEGF R2) (KDR) receptor. Downregulation of VEGF R2 has been shown in animal models to produce emphysema [89] and such downregulation of VEGF R2 has been shown to occur in emphysematous lungs [89]. Studies have also shown that the apoptosis/emphysema produced by VEGF inhibition in animal models is associated with increased markers of oxidative stress and is prevented by antioxidants [90], suggesting that oxidative stress is involved in this process but also in the systemic effects, such as muscle dysfunction in COPD [91].

ANTIOXIDANT THERAPY

Although there is now convincing evidence for an oxidant/antioxidant imbalance in COPD, proof of concept of the role of oxidative stress in the pathogenesis of COPD will come from studies with effective antioxidant therapy.

Various approaches have been tried to redress the oxidant/antioxidant imbalance. One approach is to give effective anti-inflammatory therapy to reduce lung inflammation, which will in turn decrease oxidative stress. Possible therapeutic options for this are drugs to prevent leukocyte influx into the lungs, either by interfering with the adhesion molecules necessary for migration, 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 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. Recent preliminary studies with cilomilast, phosphodiesterase 4 inhibitor, have shown some therapeutic benefit in patients with COPD [92]. The mechanism by which such drugs may act is by increasing cAMP, which decreases neutrophil activation. In particular, the release of ROS by neutrophils may be decreased, since increasing cAMP blocks the assembly of NADPH oxidase [93]. In vitro studies have shown that Cilomilast can indeed decrease ROS [94].

There are various options to enhance the lung antioxidant screen. One approach would be to use specific spin traps, such as α-phenyl-N-tert-butyl nitrone, to react directly with reactive oxygen and reactive nitrogen species at the site of inflammation. However, considerable work is needed to demonstrate the efficacy of such drugs in vivo. Inhibitors that have a double action, such as the inhibition of lipid peroxidation and quenching radicals, could be developed [95]. Another approach could be the molecular manipulation of antioxidant genes, such as GSH peroxidase or genes involved in the synthesis of GSH, such as γ-glutamylcysteine synthetase, or by developing molecules with activity similar to these antioxidant enzymes.

Several classes of low molecular weight antioxidant mimetics have been produced with high rate constants for enzymatic scavenging of free-radical species.

Recent animal studies have shown that recombinant SOD treatment can prevent the neutrophil influx to the airspaces and IL-8 release induced by cigarette smoking through a mechanism involving down regulation of NF-κB [86].

One of the low molecular weight mimetics of SOD has recently been shown to prevent the formation of emphysema in response to VEGF receptor blockade [96]. A further SOD mimetic of the manganoporphyrin class has also been shown in an animal model to significantly reduce the inflammation and squamous metaplasia induced by tobacco smoke [97]. Other strategies for reducing oxidative stress, such as the use of inducible nitric oxide synthase inhibitors to prevent the formation of peroxynitrite, could also be effective in preventing airway and lung inflammation.

This holds great promise if compounds can be developed with antioxidant enzyme properties that may be able to act as novel anti-inflammatory drugs by regulating the molecular events in lung inflammation.

A further 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 [98–100]. The results of these studies have been rather disappointing, although the antioxidant vitamin E has been shown to reduce oxidative stress in patients with COPD [101]. Attempts to supplement lung GSH have been made using GSH or its precursors [102]. GSH itself is not efficiently transported into most animal cells and an excess of GSH may be a source of the thionyl radical under conditions of oxidative stress [103]. Nebulised GSH has also been used therapeutically, but this has been shown to induce bronchial hyperreactivity [104]. The thiol cysteine is the rate-limiting amino acid in GSH synthesis [105]. Cysteine administration is not possible, since it is oxidised to cystine, which is neurotoxic [105]. The cysteine-donating compound N-acetylcysteine (NAC) acts as a cellular precursor of GSH and is 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 [106, 107]. NAC, given orally in low doses of 600 mgs per day to normal subjects, results in very low levels of NAC in the plasma for up to 2 h after administration [108]. After 5 days of NAC 600 mg three times daily, there was a significant increase in plasma GSH levels; however, there was no associated significant rise in BAL GSH or in lung tissue [109]. 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.

Recent meta-analyses of the effects of NAC on exacerbations of COPD have shown positive effects [109, 110], but this was not reflected in a recent randomised controlled trial of NAC in...
COPD patients, although there appeared to be some effect on exacerbations in a subgroup of patients who were not taking inhaled corticosteroids [111]. A study of N-isobutyryllysysteine, a derivative of N-acetylcysteine, also failed to reduce exacerbation rates in patients with COPD [112].

N-acystelyn (NAL) is a lysine salt of NAC. It is also a mucolytic and oxidant thiol compound, which in contrast to NAC, which is acidic, has a neutral pH. NAL can be aerosolised 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 [113] and inhibited H2O2 and O2− anion release from neutrophils harvested from peripheral blood from smokers and patients with COPD [114].

Molecular regulation of glutathione synthesis, by targeting γ-glutamylcysteine synthetase, has promise as a means of treating oxidant mediated injury in the lungs. Recent work by MANNA et al. [115] has shown that recombinant γ-glutamylcysteine synthetase in rat hepatoma cells completely protected against the tumour necrosis factor alpha-induced activation of nuclear factor-κB, activator protein-1 and the apoptosis/inflammatory process [115]. Cellular glutathione may be increased by increasing γ-glutamylcysteine synthetase activity, which may be possible by gene transfer techniques, although this would be an expensive treatment that may not be considered for a condition such as chronic obstructive pulmonary disease. However, knowledge of how γ-glutamylcysteine synthetase is regulated may allow the development of other compounds that may act to enhance glutathione.

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