

Protein oxidation at the air-lung interface

Review Article

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Summary. Whilst performing its normal functions the lung is required to deal with a range of toxic insults. Whether these are infectious agents, allergens or air pollutants they subject the lung to a range of direct and indirect oxidative stresses. In many instances these challenges lead to oxidative alterations of peptides and proteins within the lung. Measurement of protein oxidation products permits the degree of oxidative stress to be assessed and indicates that endogenous antioxidant defences are overwhelmed. The range of protein oxidation products observed is diverse and the nature and extent of specific oxidation products may inform us about the nature of the damaging ROS and NOS. Recently, there has been a significant shift away from the measurement of these oxidation products simply to establish the presence of oxidative stress, to a focus on identifying specific proteins sensitive to oxidation and establishing the functional consequences of these modifications. In addition the identification of specific enzyme systems to repair these oxidative modifications has led to the belief that protein function may be regulated through these oxidation reactions. In this review we focus primarily on the soluble protein components of within the surface liquid layer in the lung and the consequence of their undue oxidation.

Keywords: Respiratory tract lining fluid – Lung – Protein carbonyl – Air pollution – α -tocopherol – Ascorbic acid – Glutathione

Abbreviations: ARDS, Acute Respiratory Distress System; BAL, Bronchoalveolar lavage; CF, Cystic Fibrosis; COPD, Chronic Obstructive Pulmonary Disease; DHA, Dehydroascorbate; EPO, Erythropoietin; ERK, Extracellular signal-regulated kinase; GSH, Reduced Glutathione; GSSG, Oxidised Glutathione; HIV, Human Immunodeficiency Virus; MPO, Myelo peroxidase; NOS, Nitric oxide synthase; NL, Nasal lavage; ROS, Reactive oxygen species; RNS, Reactive nitrogen species; RTLF, Respiratory Tract Lining Fluid; SOD, Superoxide dismutase; SP-A, Surfactant Protein-A; 3-NT, 3-Nitrotyrosine; UA, Uric acid

Introduction

The lung, like the skin (Thiele, 2001) and the surface of the eye (Rose et al., 1998), presents the first physical interface between the outside environment and the body. As such it

is susceptible to a range of environmental toxicants, both viable, such as bacteria, viruses, allergen and fungi, and non-viable, pollutant oxidant gases (ozone, nitrogen dioxide) and airborne particulate matter. Whilst these toxicants are diverse, in all cases their pathophysiological action on the lung seems to involve the imposition of an oxidative stress (the excess generation of reactive oxygen species (ROS) in relation to available antioxidant defences). This may occur in the case of oxidant gases through the direct oxidation of biomolecules at the surface of the lung (Pryor, 1994), or in the case of micro-organisms because they engender airway inflammation (Morcillo et al., 1999; Babor, 2000). All pulmonary conditions where inflammation has been implemented (asthma, COPD, cystic fibrosis) appear to involve a component of oxidative stress. In this review we examine the impact of these challenges on the lung, focusing specifically on protein oxidation events within the thin amphipathic layer overlaying the respiratory epithelium: the respiratory tract lining fluid (Bastacky et al., 1995; van der Vliet et al., 1999). In this context the endogenous antioxidant defences within this compartment will be outlined, as are the adaptive antioxidant pathways which function to mitigate against oxidative injury. Central to our review of the current literature will be the issue of whether protein oxidation is simply a biomarker of oxidative stress (allowing some discrimination of the radical species involved) or actually a mechanistically significant event, with major impacts on protein function and lung physiology. Whilst a considerable body of work has been performed on pulmonary tissues, examining protein oxidation in a variety of disease settings, comparatively little

work has focused on protein modifications within the RTLTF. This, allied to the critical position of this compartment suggests that identification of oxidatively modified proteins within this compartment is likely to represent a fertile area of future research. Whilst much of the focus of this review will be on the soluble protein components of RTLTF we will also deal with the oxidation of proteins within lung tissue itself.

The respiratory tract lining fluid

This aliphatic compartment consists of a two-layer structure, comprising a lower aqueous sol phase and an upper mucus, gel phase. The gel phase is largely composed of high molecular weight mucopolypeptide glycoproteins derived from sub-mucosal glands (Quinton, 1979). Whilst these compounds may act as sacrificial antioxidants *in vivo* due to their thiol content (van der Vliet and Cross, 2001) their main function is to trap microorganisms and large particles from the air-stream. These particles are then transported by mucociliary action to the posterior pharynx and swallowed; therefore acting as a mechanical clearance mechanism to limit the penetration of inhaled toxicants into the lung. As a consequence of this action the gel phase of the RTLTF is continually turning over. The lower, sol phase which has been shown to have a wide range of antioxidant defences (van der Vliet et al., 1999; Cantin et al., 1987; Mudway et al., 2001) in contrast turns over relatively slowly, permitting secreted antioxidants to concentrate within this compartment (Cantin et al., 1987). In the distal airways, where mucus-secreting cells are absent, this layer is the first physical interface inhaled materials come into contact with. Furthermore, in airway inflammation the majority neutrophil, eosinophil, and macrophage actions occur within this compartment. Therefore much of the discussion in this review focusing on the antioxidant defences within the RTLTF relates to events occurring with the sol compartment. Not all aspects of lung lining fluid are currently understood. For example, although it is appreciated that the depth of the fluid layer varies along the respiratory tree, its precise thickness is still under debate (Duneclift et al., 1997; Widdicombe et al., 1997). In the upper airways, lung-lining fluid may be 1–10 μm thick compared with 0.2–0.5 μm in the distal bronchoalveolar regions. In addition whether the RTLTF comprises a continuous sheet covering the pulmonary epithelium throughout its length, as supported by high resolution EM-studies (Bastacky et al., 1995), or is patchy toward the more distal structures (Pryor et al., 1995) remains a point of contention. Both

of these issues are critical in understanding the nature of oxidation reactions occurring at the lung surface as the RTLTF may prevent or limit the extent of the reaction of inhaled toxicants with the lung epithelium.

RTLTF can be obtained from human volunteers and patients using a variety of bronchoscopy based techniques, bronchial wash and bronchoalveolar lavage (BAL). More recently the use of less invasive techniques have also become widely employed: nasal lavage, (Noah et al., 1995; Housley et al., 1995), sputum induction with hypertonic saline (Nordenhall et al., 2001; Jones et al., 2001), and the condensation of exhaled breath (Antczak et al., 2002). There is currently little data to demonstrate the quantitative relationship between indirect proximal airway measures of pulmonary inflammation with the more traditional gold standard bronchoscopy based techniques. Despite this important proviso, these less invasive techniques have permitted more detailed time course studies in human investigations, and investigation of inflammatory responses in patients with severe airways disease such as asthma and COPD. These procedures, with the exception of the collection of breath condensate yield both cell pellets and dilute samples of the RTLTF after separation of these components by centrifugation. In addition bronchoscopy based techniques often incorporate the collection of bronchial biopsies. These samples can therefore all be analysed for antioxidant concentrations and a range of oxidation markers. Collection and analysis of the RTLTF in lavage fluid supernatants however presents a number of problems: First, the RTLTF proteome may be contaminated with intra-cellular and plasma proteins if the lavage, or post aspiration processing is too robust. Second, prolonged saline, or PBS dwell times in the lung can result in erroneously high antioxidant concentrations, as these molecules move down a concentration gradient from cellular stores. Finally, unless lavage is performed rapidly, pH and contaminating transition metal sources are tightly controlled and samples are processed and stored appropriately, RTLTF components may become oxidised due to the lavage procedure.

The RTLTF proteome

The protein constituents of the RTLTF have been reviewed in detail previously (Hatch, 1992), however recent developments in proteomics using 2D-gel maps of lavage fluid samples have produced a fuller picture (Noel-Georis et al., 2002; Lenz et al., 1993; Sabounchi-Schutt et al., 2001; Lindahl et al., 1995; Wattiez et al., 1999). These techniques have allowed the identification of protein profiles

characteristic in a range of pulmonary diseases (sarcoidosis, idiopathic pulmonary fibrosis, hypersensitivity pneumonitis) and following acute airway insults (cigarette smoke) (Wattiez et al., 1999; Lindahl et al., 1996; Lenz et al., 1993; Lindahl et al., 1998; Lindahl et al., 1999a, b). BAL protein components are diverse and derived from a variety of sources: local synthesis and export by epithelial and resident inflammatory cell populations and via transduction of plasma proteins across the blood-air barrier. Proteins constitute approximately 0.5–1% of the dry weight of bronchial secretions (Creeth, 1978), and total protein concentrations in the RTLF have been reported to be in the order of 3–5 mg/mL in healthy human subjects (Rennard et al., 1990; Thompson et al., 1990). These values are derived from BAL-fluid concentrations corrected for lavage dilution using the urea correction. Whilst there are acknowledged problems with this dilutional control (Haslam et al., 1999), this is the most widely used approach in the literature and does give a indication of overall dilution, as long as the lavage dwell time is short (van der Vliet et al., 1999).

The major protein constituents of RTLF are albumin (50–60% of the total protein pool), transferrin (5–6%), α 1-antitrypsin (3–5%) and the immunoglobulins A and G (30%) (Hatch, 1992; Bell et al., 1981; Blomberg et al., 1999). The ratio of these major protein species varies depending on the compartment sampled, with albumin concentrations lower (12–20%), and lysozyme (15–35%) and lactoferrin (2–4%) concentrations higher in the nasal and bronchial RTLFs. These regional differences are also apparent in the expression of specific proteins, such as surfactant proteins in the distal lung, and mucins in the upper airways. Proteomic analysis by the group of Bernard has identified over 1200 silver stained spots, corresponding to approximately 78 protein species on 2D-gels of BAL-fluid proteins (Wattiez et al., 1999). Classification of this large number of proteins has been made on the basis of their origin: proteins derived from the plasma pool via transduction onto the surface of the lung, proteins synthesised in the lung, but also present in the plasma, and proteins unique to the surface of the lung. Using this approach it has been shown that the vast majority of the proteins found within the nasal RTLFs are derived from the plasma, whilst the distal lung has a significant complement of lung specific proteins (Noel-Georis et al., 2002). BAL proteins can also be classified according to their function: proteases/anti-proteases (α -1-trypsin, α -1-Antitrypsin, α -1 antichymotrypsin, α -1-antiplasmin, α -1-antiprotease, α 2-Macroglobulin, secretory leukoprotease inhibitor); antioxidant enzymes (AOEB166, superoxide dismutase,

glutathione S transferase, thioredoxin, plasma glutathione peroxidase), metal-chelating proteins (transferrin, lactoferrin, ceruloplasmin); surfactant proteins (SP-A/B/C and D), proteins involved in tissue repair and cellular proliferation (fibronectin, calyculin, calgizzarin, cathepsin D, zinc- α -2 glycoprotein, transthyretin, clusterin, intestinal trefoil factor, α 2-HS-glycoprotein); regulating airway immune responses (IgG, IgA, Ig-binding protein, complement fragments 3 and 4, lipocortin-1, Clara cell protein 16, lipocalin, α 1-acid glycoprotein); anti-microbial proteins (defensins, cystatin S); lipid metabolism (acyl-CoA-binding protein, FABP-E, FABP-A, saposin D); and calcium binding (calvasculin, calreticulin, calgranulin A). Detection of certain proteins by this method is problematic due to issues of hydrophobicity, low molecular mass of certain proteins and also low expression levels. In addition some of the proteins detected are clearly intra-cellular (γ - and β -actin, tubulin β 2, tropomyosin, haemoglobin) suggesting cellular lysis. This list is far from exhaustive and does not include the wide range of cytokines/chemokines (IL-1/4/5/6/8/10/18, TNF- α Gro- α , Rantes) and inflammatory cell activation markers (myeloperoxidase, eosinophil peroxidase, eosinophil cationic protein, and s-Selectin) that have been detected in BAL-fluid, both basally and during inflammatory episodes.

Antioxidant defences within the RTLF

Reactive oxygen (hydroxyl radicals, superoxide, and hydrogen peroxide) and nitrogen species (nitric oxide, nitrite, and peroxynitrite) are produced in the body as a part of normal metabolism: through leakage from electron transport chains, as by-products of a number of enzyme reactions (cytochrome-P450, liposoygenase, cyclooxygenases, peroxidases), and through secretion from activated leukocytes. Whilst these species may play a modulating role in a range of physiological functions (Conner and Grisham, 1996), over production in a range of conditions has been shown to cause tissue injury (Repine et al., 1997). Endogenous antioxidant defences offset the potential damaging action of these reactive oxygen and nitrogen species. These may either act to directly detoxify these species or by converting them to less damaging species that can be removed by other components of the antioxidant network. However, if these defences are overwhelmed damage to cellular components will occur. By virtue of its large surface area and daily exposure to inhaled toxins the lung is particularly susceptible to oxidative and nitrosative injury. A number of other unique features of this compartment may also predispose it to oxidative injury. First, there is evidence, that the RTLF,

unlike virtually every compartment in the body has a significant pool of non-transferrin bound iron (Gutteridge et al., 1996). Second, this extracellular compartment is poorly invested with antioxidant enzyme defences compared with intra-cellular levels. Although both extra-cellular SOD (Mudway et al., 2001) and plasma glutathione peroxidase (Avissar et al., 1996) have been reported in the RTLF of healthy subjects, catalase and glutathione reductase are absent. Compounding this is the high concentration of peroxidase-like enzymes within this compartment: lactoperoxidase, myeloperoxidase and eosinophil peroxidase.

To offset this considerable burden the lung has evolved a range of specialized antioxidant defences within the RTLF (Heffner and Repine, 1989). These not only include the enzymatic antioxidants described in the previous section but also a range of small molecular weight antioxidants that act as direct free radical scavengers. It is important at this point, and in the context of expanding this discussion into the oxidation of proteins to state that the RTLF antioxidant network should not however be thought of as a static pool opposing any imposed oxidative insult. Instead the defences within this compartment should be thought of as a dynamic equilibrium with the antioxidant defences within the epithelium and the more remote plasma pool. The most characteristic features of all oxidative challenges are the range of adaptive antioxidant responses observed; either through increased synthesis (Rahman and MacNee, 2000) or the enhanced movement of reducing equivalents to the RTLF (Freed et al., 1999). A fine balance therefore exists between basal antioxidant levels, oxidant fluxes and adaptive modifications to antioxidant reserves all of which modulate the capacity of ROS and RNS to oxidise protein amino acids.

Ascorbic acid

As a consequence of its high water solubility, L-ascorbate (vitamin C) is widely distributed throughout the body, including the RTLF. Ascorbate is an excellent reducing agent and scavenges a variety of free radicals and oxidants *in vitro*, including superoxide, peroxy radicals, hydrogen peroxide, hypochlorous acid, singlet oxygen, ozone (Mudway and Kelly, 1998; Frei et al., 1989; Langford et al., 1995) and nitrogen dioxide (Kelly and Tetley, 1997; Postlethwait et al., 1995). During this scavenging activity, ascorbate loses one electron resulting in the formation of the semi-dehydroascorbate radical (Buettner and Jurkiewicz, 1996), which rapidly decomposes to dehydroascorbate and a range of further oxidation products, terminating in oxalate. As oxalate is cytotoxic, most cells contain a variety of dehy-

droascorbic reductase enzymes that catalyse the regeneration of ascorbate from dehydroascorbate at the expense of GSH and NADPH (Wang et al., 1997; Washburn and Wells, 1999; May et al., 1997). Notably, whilst a range of enzymes has been reported to have DHA-reductase activity intracellularly (glutaredoxin, NADPH-dependent-3 α -hydroxysteroid DHA reductase, erythrocyte glutathione peroxidase) only glutathione peroxidase has been reported in the RTLF. GSH can regenerate ascorbate from DHA non-enzymatically (Winkler et al., 1994) but again this is unlikely at RTLF concentrations. These two observations suggest that the ascorbate pool on the surface of the lung is likely to be highly labile, a contention further supported by the presence of non-transferrin bound iron in this compartment (Gutteridge et al., 1996), and basal hydrogen peroxide concentrations (Kharitonov and Barnes, 2002). Thus maintenance of adequate ascorbate concentrations at the lung surface require it to be rapidly transported from cellular sources or from the plasma pool. Which of these pathways occurs is currently not understood but absence of a clear association between plasma and lavage fluid ascorbate concentrations (over a wide range of plasma concentrations, 20–100 μ M) suggests that simple diffusion from the plasma pool does not explain RTLF levels. Further, undiluted RTLF concentrations have been reported at 100–400 μ M (Cantin et al., 1987; van der Vliet et al., 1999) above plasma saturation levels, suggesting that a simple diffusion gradient does not exist. In addition to the direct scavenging action of ascorbate it also acts indirectly to prevent lipid peroxidation through its reaction with membrane-bound tocopherol. In *in vitro* studies, it has been demonstrated that ascorbate is able to reduce the tocopherol radical back to tocopherol, thereby restoring its scavenging activity (Halpner et al., 1998). This synergistic action of ascorbate though clearly demonstrated *in vitro*, has importantly, not yet been conclusively demonstrated *in vivo*. Whilst ascorbate has many antioxidant actions, it also has the capacity to act as a pro-oxidant in the presence of transition metals. There is now a considerable body of evidence indicating that much of the toxicity attributed to inhaled airborne particles may be due to the presence of transition metals absorbed onto their surface (Ghio and Devlin, 2001). One might therefore speculate that these particles may actually subvert much of the protective character of the RTLF, with ascorbate acting as a pro-oxidant, resulting in tissue injury, instead of protecting against it.

Glutathione and glutathione enzymes

RTLF contains high concentrations of glutathione (L- γ -glutamyl-L-cysteinylglycine) at concentrations approximately

100 times greater than those in plasma, and in a predominantly (>90% GSH) reduced form (Cantin et al., 1987). Initial estimates of the concentration of GSH in undiluted RTLF were approximately 400 μ M (Cantin et al., 1987 #869), but concentrations are now considered to range between 100–200 μ M (van der Vliet et al., 1999), with the concentration of GSSG being critically dependent on the magnitude of airway inflammation, but typically <90% of the total pool (Cantin et al., 1987). A number of cell types, alveolar macrophages, Clara cells and alveolar Type II cells have been reported to contain high levels of glutathione (4.8, 3.2 and 0.54 mM, respectively (Horton et al., 1987). Poor re-absorption of glutathione from the respiratory tract is also important. The cell surface enzyme responsible for the uptake of glutathione from the extra cellular space, γ -glutamyl transpeptidase is present in much lower concentrations in the lung compared with other organs but may be located in specific epithelial cells such as Clara and Type II cells (Dinsdale et al., 1992). RTLF is also replenished relatively slowly therefore any glutathione exported into this compartment is likely to remain there for prolonged periods. Glutathione is particularly good at defending against inflammatory cell derived oxidants such as superoxide, hypochlorous acid and hypobromous acid (Winterbourn, 1985). Though GSH concentrations are high in the RTLF, the glutathione redox cycle is not operative in this compartment due to the absence of glutathione reductase. Thus any GSSG formed either directly through the reaction of GSH with ROS/RNS, or through the detoxifying actions of glutathione peroxidase will tend to accumulate. Whilst glutathione reductase has been reported in lavage in some studies its presence has generally been taken as evidence of cell lysis. Therefore glutathione probably functions more in a sacrificial mode in the RTLF, reacting directly with ROS with the production of thiyl radicals (GS $\cdot$) which can subsequently be converted to glutathione disulphide (GSSG) through a radical transfer process. Thiyl radicals should not however be considered wholly harmless as they have been shown to react with other targets propagation free radical induced injury (Pryor, 1994; Kalyanaraman, 1995).

The concentration GSH has been shown to be altered in a range of pulmonary conditions, either decreased, in asthma during exacerbations (Comhair et al., 2000), idiopathic pulmonary fibrosis (Cantin et al., 1989), asbestosis (Brown et al., 2000), acute respiratory distress syndrome (Bunnell and Pacht, 1993) and in HIV positive patients (Pacht et al., 1997); or increased, in chronic smoking (Cantin et al., 1987), chronic beryllium disease (Comhair et al., 1999), or following exposure to ozone (Blomberg et al., 1999) and nitrogen dioxide (Kelly et al., 1996).

In addition, the concentration and activity of plasma glutathione peroxidase is also influenced by oxidative stress. Its activity in the RTLF of smokers is increased (Melloni et al., 1996), while decreased activities are seen after ozone exposure (Avissar et al., 2000), and unaltered activities following exposure to nitrogen dioxide (Avissar et al., 2000). Recent studies have also provided evidence for GSH-dependent enzymes glutathione S transferase and thioredoxin within the RTLF (Noel-Georis et al., 2002). The role of these two enzymes within this compartment has not currently been investigated in human subjects. Glutathione S transferases represent a family of enzymes responsible for the detoxification of endobiotic and xenobiotic compounds by covalently linking them to glutathione. It is therefore possible that they may play a significant role in the detoxification of organic radicals inhaled in cigarette smoke (Squadrito et al., 2001) and introduced into the lung on the surface of inhaled particulates (Dellinger et al., 2001). This function if operative in the RTLF could explain the reported loss of GSH in certain acute lung insults where there is no evidence of an increase in GSSG. Thioredoxin catalyses thiol disulphide exchange reactions and is therefore critical in the regulation of protein function by thiol redox control. Given the propensity for numerous RTLF proteins to undergo thiol modification under oxidative stress, investigations into the regulation of this enzyme in a number of pulmonary conditions is clearly warranted.

Uric acid

Uric acid is also an important antioxidant in RTLF (Ames et al., 1981) especially in the upper airways where its concentration is high (Peden et al., 1990). Uric acid is an oxidised purine base which is an effective scavenger of hydroxyl radicals, ONOO $^-$, and oxyhaem oxidants formed between the reactions of haemoglobin and peroxy radicals, peroxy radicals themselves and singlet oxygen (Becker, 1993). It acts in these reactions in a sacrificial mode, in that it is irreversibly damaged through the interaction producing a range of oxidation products including allantoin (Ames et al., 1981). It is also a highly effective scavenger of both inhaled ozone (Mudway et al., 1996; Mudway et al., 1999) and nitrogen dioxide (Kelly and Tetley, 1997). From our observations to date, we have noted that RTLF uric acid concentration seems to be associated closely with protein concentration. This seems to be the case at all levels of the respiratory tract. This finding infers that RTLF uric acid comes from either a direct flux from the plasma pool (Becker, 1993; Housley et al.,

1995), or alternatively some UA may be stored and then actively secreted from sub-mucosal glands (Peden et al., 1993).

α -Tocopherol

α -Tocopherol (vitamin E) is present within RTLF, albeit at relatively low concentrations: 50–200 nM. It is thought that this α -tocopherol is secreted by type II cells in to RTLF along with surfactant (Rustow et al., 1993). α -Tocopherol is a powerful antioxidant, both in terms of its direct free radical scavenging activity, and through its ability to terminate lipid peroxidation (Burton et al., 1981; Burton et al., 1983). Vitamin E functions as a chain-breaking antioxidant in the lipid phase. Evidence of this function is however indirect, focusing almost entirely on observations made using *in vitro* systems (Burton et al., 1983). It is thought that reactivity with organic peroxy radicals accounts for the majority of the biological activity of α -tocopherol (Burton et al., 1981). This reaction is of considerable importance because tocopherols reacting with lipid peroxy radicals yield a relatively stable lipid hydroperoxide and a vitamin E radical, which effectively interrupts the lipid peroxidation chain reaction (McCay, 1985).

Extra-cellular SOD

Whilst 2D-Gel maps have reported the presence of Mn-SOD (Noel-Georis et al., 2002) in RTLF it is likely that this antioxidant enzyme is present as a contaminant due to cell lysis during lavage and lavage fluid processing. RTLF has also been shown to contain appreciable concentrations of extra-cellular SOD, both in nasal (Mudway et al., 1999) and BAL-fluid samples (Mudway et al., 2001) presumably closely associated with proteoglycan at the epithelial surface. The association of this antioxidant enzyme with epithelial and endothelial surfaces may be significant as it may function to limit the production of peroxynitrite in local environments in which superoxide and nitric oxide are produced.

Metal chelating proteins

The RTLF has also been shown to contain a range of metal binding proteins: caeruloplasm, lactoferrin, transferrin, (Pacht and Davis, 1988; Noel-Georis et al., 2002). These chelators perform an important antioxidant function *in vivo* by regulating free iron concentrations and hence limiting the potential for Fenton-like chemistry, with the formation of the damaging hydroxyl radical. Despite detectable concentrations of these proteins in lavage fluid from healthy control

subjects (Gutteridge et al., 1996), and evidence of their upregulation during a variety of pulmonary conditions and acute oxidant insults (Gutteridge et al., 1996; Nelson et al., 1996; Ghio et al., 1998), there is evidence that RTLF contains a significant 'free' non-transferrin bound iron pool (Gutteridge et al., 1996; Nelson et al., 1996), which is available for potential redox reactions.

Distribution of antioxidants within the respiratory tract

Although it is clear that there are differences in antioxidants between different regions of the respiratory tract, the extent of these differences has not yet been clarified. In the nasal cavity, uric acid is by far the most prevalent antioxidant (Peden et al., 1990; Housley et al., 1995). It should be noted however that there are gender differences with women having significantly lower levels of uric acid than men (Housley et al., 1996). A similar gender difference exists for plasma concentrations of uric acid, indicating that the major source of this antioxidant in NL is from plasma. However, Peden et al. demonstrated that in the nasal cavity, RTLF Sol phase uric acid concentration is increased following cholinergic stimulation of the airways (Peden et al., 1991). Furthermore, they found that the increase in uric acid correlated positively with lactoferrin concentration, but not with albumin. As lactoferrin is predominately derived from mucosal gland secretions (Gel phase) this supports the contention that RTLF uric acid in the upper airways is derived, in part, from glandular secretions. Control of uric acid secretion in this compartment may therefore be closely related to mucus secretion. Glutathione and ascorbic acid are also present in nasal cavity RTLF however at significantly lower concentrations. Furthermore, although not always the case, low concentrations of α -tocopherol are sometimes observed. In the peripheral airways (BL fluid) and bronchoalveolar region (BAL fluid) uric acid, ascorbic acid, GSH and α -tocopherol are usually present.

ROS and RNS generation in the RTLF

The respiratory tract is exposed to a variety of ROS and RNS, predominately through exposure to exogenous oxidants (ozone, nitrogen dioxide) and inflammation. During inflammatory processes a number of cell types can release ROS and RNS. Neutrophils, eosinophils, B-lymphocytes and monocytes (but not macrophages, except under special circumstance) all contain the NADPH oxidase enzyme system which catalyses the production of superoxide from

oxygen and NADPH (Babior, 2000). In phagocytic cell populations the superoxide, released into the extra-cellular environment by this enzyme, is involved in the destruction of microorganisms. Superoxide is relatively unstable, though unreactive (redox potential -330 mV) radical, which will dismutate to hydrogen peroxide, in SOD catalysed and un-catalysed reactions. Whilst superoxide is relatively unreactive it will participate in the liberation of Fe^{2+} from ferritin (Harris et al., 1994), and aconitase (Gardner et al., 1995) with a loss of protein function and release potential dangerous 'free'-iron. Ultimately therefore hydrogen peroxide is the predominate oxidant formed by phagocytes. Hydrogen peroxide is also a relatively weak oxidising species, which allied to cellular glutathione peroxidase and catalase defences means its toxicity *in vivo* is not thought to be significant. Hydrogen peroxide can also be produced directly by a variety of oxidase enzymes including xanthine oxidase, monoamine, and amino acid oxidase. The damaging actions of hydrogen peroxide are therefore predominately related to its reduction to a variety of more damaging oxidant species. Hydrogen peroxide is a key substrate for the neutrophil derived myeloperoxidase, and the eosinophil derived, eosinophil peroxidase enzymes which catalyses the oxidation of halide anions (MPO, Cl^- , Br^- and I^- ; EPO, Br^- and I^- only) to form hypochlorous (HOCl) and hypobromous acid (HOBr) (Foote et al., 1983; Thomas et al., 1995). These highly reactive oxidants have been shown to react with thiols, and thioethers (Vissers and Winterbourn, 1995), halogenate nucleotides, and with amine groups to form halamines which may have even greater reactivity than the parent radical (Thomas et al., 1983).

Hydrogen peroxide can also undergo a transition metal catalysed reduction to the highly damaging hydroxyl radical. A range of biological reductants including ascorbate can then reduce the metal oxidised in this reaction. This reaction is critically dependent on the presence of 'free' Fe^{2+} and Cu^+ . *In vivo*, these metals are typically sequestered into a range of proteins: ferritin, transferrin, lactoferrin, ceruloplasmin etc. Therefore whether this is a significant pathway *in vivo* is questionable. Hydroxyl radicals can also be formed in the reaction between HOCl and superoxide but again the significance of this pathway *in vivo* has not been fully established (Kettle and Winterbourn, 1994). Whilst the hydroxyl radical is highly reactive, not only does its formation require the presence of 'free' or unchelated iron, but such is its rate of reaction with a broad spectrum of organic molecules that its toxic action will tend to be diluted out by its very promiscuity. Therefore, whilst undoubtedly highly reactive, the hydroxyl radical is

more likely to interact with non-vital cellular components than critical targets.

The hydrophobic gas nitric oxide (NO) is produced endogenously *in vivo* where it plays a role in a range of important functions in the maintenance of normal airway physiology and host defense: modulating the expression of inflammatory cytokines, adhesion molecules and enzymes of the eicosanoid biosynthetic, as well as having a modulating influence on a range of antioxidant systems (Halliwell et al., 1999; Brennan and Moncada, 2002). Nitric oxide is produced from L-arginine by a variety of cells within the airway, both epithelial (type II pneumocytes) and inflammatory (macrophages, neutrophils, mast cells) through the action of constitutive (Ca^{2+} dependent, short term, low capacity) and inducible (Ca^{2+} independent, long term, high capacity) forms of nitric oxide synthase. The expressions of these enzymes are increased during inflammation with concomitant increases in exhaled NO, as observed in asthmatics (Kharitonov et al., 1997), in patients with bronchiectasis, during airway infections and following inhalation of oxidant gases (Balint et al., 2001; Kharitonov and Barnes, 2002; Kharitonov and Barnes, 2000; Nightingale et al., 1999; Kharitonov and Barnes, 2001). Similarly the end products of NO metabolism, nitrite (NO_2^-) and nitrate (NO_3^-) are increased in RTLF in these conditions (Balint et al., 2001). Whilst most of the NO detected in exhaled breath is derived from the nasal airways and sinuses it is clear that NO can be produced throughout the length of the airway. Although a free radical itself, NO is not particularly reactive allowing it to diffuse readily *in vivo* from its site of production to its site of action. It can however react with a range of other radical species (superoxide, thiol, lipid and protein radicals) to generate a variety of highly damaging radical intermediates of considerably greater toxicity than itself.

Nitric oxide can react with oxygen to form nitrogen oxides: the powerful oxidant nitrogen dioxide (NO_2), as well as nitrite (NO_2^-) and nitrate (NO_3^-) but these reactions are slow and not favoured *in vivo* (Beckman and Koppenhol, 1996). Instead much interest has focused on the rapid reaction between NO and superoxide (out competing its dismutation by SOD) with the formation of the highly damaging oxidant species: peroxynitrate (ONOO^-) (Beckman et al., 1994). It is also clear that both the inflammatory cells of the airways and the epithelium itself are capable of producing both NO and superoxide (Munoz-fernandez et al., 1992). It is now believed that much of the toxicity previously ascribed to the hydroxyl, superoxide and nitric oxide *in vivo* is now often attributed to peroxynitrite and its derivatives (Tien et al., 1999). The ability

of ONOO^- to nitrite proteins as well as induce lipid peroxidation and DNA damage has been demonstrated *in vitro*. In contrast to the hydroxyl radical the rate with which peroxynitrite will react with biomolecules is rather slow, favouring selective reactions with its major targets: sulphhydryls (Stamler et al., 1992), iron/sulphur centres (Hausladen and Fridovich, 1994) and zinc-fingers. Peroxynitrate is surprisingly uncreative toward reduced glutathione and is therefore not an effective scavenger for this radical. Indeed unlike most oxidants, peroxynitrite will oxidize protein targets without having to first deplete endogenous antioxidant defences (van der Vliet et al., 1994). This potent species will participate in a wide range of oxidation reactions including the oxidation of protein and non-protein sulphhydryls, deoxyribonucleic acid and lipids, as well as nitration of tyrosine residues by catalysed (low molecular weight transition metals and superoxide dismutase) and un-catalysed reactions.

Peroxynitrite will also undergo protonation in acid environments to produce the strongly oxidizing peroxynitrous acid radical, which decomposes to nitrate via intermediate formation of the hydroxyl radical and a nitrogen dioxide-like species (Beckman et al., 1990). This may represent a significant nitrating species on the surface of the lung of asthmatic subjects where RTLTF has been demonstrated to be acidic (Hunt et al., 2000). Peroxynitrite will also react rapidly with CO_2 to form nitrogen dioxide and the CO_2 adduct nitrosperoxocarbonate (ONOOCO_2^-), which has been proposed to represent the major damaging peroxynitrate derived intermediate formed under physiological conditions (Tien et al., 1999). The ability of ONOO^- to nitrite proteins as well as induce lipid peroxidation and DNA damage has been demonstrated both *in vitro* and *in vivo*. It therefore appears that peroxynitrite, and its derivatives, is one of the major damaging oxidants formed *in vivo*, and certainly accounts for much of the toxicity associated with the increased production of nitric oxide. Notably, in the absence of these physiological catalysts the rates of nitration seen in model systems are low and indeed diminish significantly as addition components are added to the models (Ischiropoulos et al., 1992). Protein nitration does not always imply the local production of ONOO^- . Nitration by ONOO^- catalysed by MnSOD occurs through a mechanism thought to involve the cleavage of the peroxide bond to produce a nityl cation like intermediate. Due to the short half-life of this species it does not exist free.

NO can also be converted to the highly reactive NO_2 radical *in vivo* through various biological pathways: The auto-oxidation of NO, via one electron reactions with

antioxidants, thiols, aromatic amino acids, unsaturated lipids (Pryor and Squadrito, 1995) and via the one electron reduction of nitrite. In addition under acidic conditions (gastric compartment, phagosomes, tissue acidosis) nitrite can be protonated to nitrous acid (HNO_2), which can decompose to NO and NO_2 (Ohshima et al., 1990). Nitrite also acts as a substrate for MPO and EPO in a reaction producing the highly reactive NO_2 radical. High concentrations of these peroxidases can be found at sites of leukocyte activation, where they have been shown to be associated with evidence of protein nitration (Heinecke, 1997). This pathway is dependent on the concentrations of nitrite (10–140 μM in nasal and 10–15 μM in alveolar RTLTF). Significantly nitrite does not accumulate *in vivo* being further metabolised to nitrate (Parks et al., 1981) but levels do increase during inflammatory episodes.

Inhaled ozone, nitrogen dioxide and PM

The lung is also susceptible to oxidative damage from exogenous oxidant species. The secondary pollutant gas, ozone, represents an excellent example in that through its powerful oxidising ability it will react with numerous biomolecules. Despite the broad target specificity of ozone, the rate at which reactions occur with different molecules can vary over several orders of magnitude (Pryor, 1993). Indeed when ozone encounters an array of potential substrates it reacts initially with those with the highest intrinsic reactivities toward it. Uric acid, ascorbic acid and glutathione have all been shown (in defined settings) to display high intrinsic reactivities toward ozone (Pryor et al., 1995; Cross et al., 1992; Mudway and Kelly, 1998; Postlethwait et al., 1994). It has therefore been proposed that RTLTF antioxidants function as sacrificial substrates for ozone (Pryor et al., 1985). By reacting with ozone they effectively remove it with only the production of ostensibly harmless products. It should however be noted that thiyl and ascorbyl radicals are not without some biological significance. When these defences are overwhelmed oxidation to lipid and protein moieties can occur. As ozone is relatively insoluble it is unlikely that unreacted ozone can penetrate the RTLTF to directly oxidise epithelial cell membranes. Instead its toxicity is transmitted to the underlying cells through the generation of a range of lipid oxidation products (LOPs) (Pryor et al., 1995), which have been reported to have pro-inflammatory properties (Kafoury et al., 1999).

The primary gaseous pollutant nitrogen dioxide is also highly oxidizing and its uptake into the RTLTF is also coupled to its reaction with RTLTF substrates, predominantly reduced glutathione and ascorbate (Postlethwait

et al., 1991; Connor et al., 2001). Because absorption is directly coupled to these reactions the products clearly must drive the events leading to tissue injury. The initial reaction products of these reactions are the thiyl and ascorbyl radicals and nitrite. Thiyl and ascorbyl radicals are able to generate ROS through a variety of mechanisms (Buettner and Jurkiewicz, 1993; Miller and Aust, 1989; Scarpa et al., 1983). Thiyl radicals may react with thiolated (GS^-) to yield superoxide and GSSG, or molecular oxygen to produce $\text{GSOO}^\cdot$ and $\text{GSO}_2\text{OO}^\cdot$ (Buettner and Jurkiewicz, 1993). The reaction between NO_2 and ascorbate can also result in the generation of oxidants: the ascorbyl radical and nitrite. Whilst the latter comprises a substrate for heme peroxidase catalysed formation of RNS, the ascorbyl radical can react with free iron, ultimately resulting in the formation of hydrogen peroxide (Miller and Aust, 1989; Scarpa et al., 1983). Therefore the reaction of both of these pollutant gases with the RTLTF can give rise to a variety of radical species.

Cigarette smoke also contains an extensive variety of free radicals and oxidant species (including NO_2), both in the gas and tar phase (Kim et al., 2002) and therefore will both introduce directly radicals to the lung surface, as well as generate them as a result of oxidation reactions at the RTLTF -air interface. Much recent work in the air pollution field has focused on the damaging role of inhaled particulate matter of an aerodynamic diameter of $<10\ \mu\text{m}$. These particles induce oxidative stress within the lung via the introduction of transition metal (Ghio et al., 1998) and stable organic radicals (Squadrito et al., 2001) into the lung. All of these exogenous agents are also capable of eliciting acute inflammatory responses in the human lung and hence a secondary source of ROS and RNS (Committee of the Environmental and Occupational Health Assembly of the American Thoracic Society, 1996).

Protein oxidation

Reactive oxygen and nitrogen species can react directly or indirectly with proteins to cause a range of oxidative modifications. The key question when addressing the significance of these modifications is whether they are simply markers for the presence of oxidative stress, or have some substantive consequence on protein function that impacts on tissue injury or disease progression. To achieve this it is important to show that the amino acid modification is related to altered function, and not simply a parallel event. Here we will describe the major oxidative modifications, which have been reported, specifically focusing on those studies where oxidation has been related to altered function. By necessity

this will require a consideration of findings derived from other organ systems, as to date only protein nitrosylation, methionine oxidation and protein carbonyl formation have been dealt with in any detail in the lung, and then mostly focusing on BAL cells or tissue biopsies. In the previous section considering the RTLTF proteome it was clear that the major proteins could be classified into four groups: those related to (a) protease/anti-protease balance, (b) antioxidant defence and metal ion chelation, (c) regulation of inflammatory response, and (d) tissue repair and remodelling. It is therefore reasonable to assume that if oxidation of these proteins in other body compartment alters function (under an appropriate stress), these findings will be of relevance to the RTLTF compartment. These types of detailed analysis in lavage fluid are still hampered by the low total protein concentrations in this compartment, though the use of proteomic technologies should help to resolve these issues.

Reactive oxygen and nitrogen species and their secondary lipid and sugar oxidation products may interact with proteins to cause oxidation of the polypeptide backbone of the protein, peptide bond cleavage, protein-protein cross-linking, and a range of amino-acid side chain modifications. All amino acids are potential targets for oxidation by reactive oxygen and nitrogen species but the derived products have only been fully characterised in a few cases. In the physiological context the major aromatic amino acids tyrosine, tryptophan, phenylalanine, the sulphur containing amino acids, cysteine, methionine, as well as the aliphatic amino acids arginine, lysine, proline, and histidine are the major targets of ROS/RNS attack. Cysteine and methionine, the two sulphur-containing amino acids appear especially sensitive to oxidation.

Methionine oxidation

Methionine is easily oxidized to methionine sulfoxide derivatives in a mechanism mediated by a wide range of ROS (chloroamines, peroxynitrite, superoxide, hydrogen peroxide, hypochlorous acid) at physiological pHs. This oxidative modification can be reversed through the action of methionine sulfoxide reductase (Brot and Weissbach, 1983). Further oxidation products of methionine sulfoxide such as the sulphone cannot be reduced to methionine. The reversible oxidation-reduction of this residue has led to suggestions that this might act as a means of regulating enzyme activity, as well as protecting vital MET residue from irreversible oxidation with a corresponding loss of protein function (Levine et al., 2000). The oxidation of methionine residues has also been shown to result in an increase in protein surface hydrophobicity (Chao et al.,

1997). This is related to the exposure of hydrophobic residues usually buried within the protein, as structural integrity is lost due to amino-acid oxidations. This increased hydrophobicity has been shown to increase the susceptibility of proteins to ligation with ubiquitin (Hershko and Ciechanover, 1986), targeting the proteins for proteolytic degradation by the proteasome.

A wide range of proteins has been shown to display altered function following methionine oxidation. Focusing on those proteins likely to be of significance on the surface of the lung, exposure of α -1-anti-trypsin to a variety of ROS *in vitro* has been shown to cause oxidation of surface exposed methionine residues at its reactive centre (met-351, met-358) associated with a loss of activity (Johnson and Travis, 1979; Taggart et al., 2000). Treatment with methionine reductase restored anti-elastase activity suggesting that the functional impairment was directly related to the methionine oxidation (Carp et al., 1983). Similarly, the proteinase inhibitor α -2 macroglobulin has been shown to possess oxidisable methionine residues (Reddy et al., 1994) though their oxidation in this case did not appear to result in a loss of antiprotease function. Instead their oxidation appeared to spare the oxidation of a critical tryptophan residue related to the protein function suggesting that in certain proteins methionine may have protective antioxidant functions. Other antiproteases have also been reported to have their function modified through oxidation of their methionine residues: α -2-antiplasmin (Geary and Gonias, 1989), mucus proteinase inhibitor (Boudier and Bieth, 1994) and secretory leukocyte proteinase inhibitor (Tomova, 1994). Methionine oxidation has also been implemented in altered function in a variety of proteins regulating the inflammatory response, present within the RTLF: Interferon γ (Keck, 1996), IL-6 (Nishimura et al., 1991), and lysozyme (Jori et al., 1968). In these studies the involvement of methionine oxidation in the modulation of protein function was generally examined in simple *in-vitro* models; whether these specific oxidations occur *in vivo* remains an open question. There is however evidence for methionine oxidation of RTLF proteins *in vivo*. Elevated concentrations of oxidised methionine residues have been detected in BAL proteins obtained from lung transplantation patients suffering from bronchiolitis obliterans syndrome (Behr et al., 2000; Hirsch et al., 1999). Increased concentrations of methionine sulfoxide have also been detected in BAL fluid obtained from patients with acute and chronic bronchitis (Maier et al., 1992), chronic obstructive pulmonary disease (Maier et al., 1992), and lung cancer patients under-going radio chemotherapy (Beinert et al., 2000). In each of these cases, the protein species that

has been modified remains unknown. The measurement of methionine sulfoxide concentrations is simply used as an index of oxidative stress. Whilst it might be possible to relate a loss of a candidate proteins function, one which is known to have altered function related to specific methionine oxidation, with generic total protein levels of methionine sulfoxide, there is still a requirement to identify the modified protein if a direct causation is to be made.

Cysteine oxidation, S-thiolation and S-nitrosation

Cysteine residues are easily oxidised resulting in enzyme inactivation, alterations in protein tertiary structure and protein cross-linking (Thomas et al., 1995). Like methionine, cysteine residues can be oxidised to their corresponding sulfoxides: sulfenic (RSOH), sulfinic (RO₂H), and sulfonic acid (RSO₃H) by a range of reactive oxygen and nitrogen species (Claiborne et al., 1999). Of these, only the formation of sulfenic acid is reversible under physiological conditions, though this species readily oxidises to irreversible oxidation products referred to above (Prutz, 1992), or reacts with GSH to form a disulphide bond (Claiborne et al., 1993). The conversion of critical cysteine residues to sulfenic acids by S-nitrosoglutathione, and other reactive nitrogen species has been implemented in the inactivation of glutathione reductase (Becker et al., 1998), and Cathepsin K (Percival et al., 1999) in *in vitro* models.

The most studied oxidative modifications of cysteine are the formation of disulphide bonds both within and between proteins and the formation of mixed disulfides with small molecular weight thiols: cysteine, GSH, homocysteine (Klatt and Lamas, 2000). Under conditions of oxidative stress these thiolation reactions occur as a result of either partially oxidised protein sulphhydryl (thiyl radicals and sulfenic acid intermediates) with thiols such as cysteine and glutathione, or by thiol/disulfide exchange reactions with GSSG (Thomas et al., 1995). Further the formation of these mixed disulphides has been shown to alter the function of a diverse range of proteins under conditions of oxidative stress: ion translocators (Shattock and Matsuura, 1993), protein kinases (Ward et al., 1998), phosphatases (Mahadev et al., 2001), antioxidant enzymes (Schinina et al., 1996), G-proteins (Mallis et al., 2001), metabolic (Cappiello et al., 1996) proteasome complexes (Demasi et al., 2001) and structural proteins (Eaton et al., 2002; Hanson et al., 1999), as well as DNA isomerases (Wang et al., 2001). These modifications are easily reversed, either through changes in intra-cellular redox, or via enzymatic reduction by thioredoxin, glutaredoxin, or disulfide isomerase at the expense of NADPH (Grant, 2001). Many studies have

shown that S-thiolated proteins formed during oxidative stress are readily de-thiolated once the stress is removed (Seres et al., 1996) associated with complete restoration of function (Ravichandran et al., 1994). It has therefore been proposed that thiolation/de-thiolation may regulate protein function in much the same way as other reversible posttranslational modifications, such as phosphorylation and adenylation. Regulatory cysteine residues are generally located in protein regions characterised by high pKa's, as these areas favour the formation of reactive thiolated ions which are sensitive to oxidative modification by a number of oxidants: GSSG, nitric oxide, nitrosothiols, peroxynitrite, hypochlorous acid, and reactive lipids (Padgett and Whorton, 1998; Mohr et al., 1999; Pullar et al., 2001; Stamler et al., 2001).

The formation of mixed disulfides has been observed in a wide range of cells and tissues exposed to oxidative stress, both *in vitro* and *in vivo*. Mixed disulphides have been reported in rat lung tissue after exposure to cigarette smoke (Park et al., 1998) and hyperoxia (Awasthi et al., 1998), in the human lens during cataract formation (Lou et al., 1999), in models cadmium-induced oxidative stress (Figueiredo-Pereira et al., 1998), and during ischemia/reperfusion in isolated rat hearts (Eaton et al., 2002). Further, stimulation of respiratory burst in isolated human neutrophils (Seres et al., 1996; Chai et al., 1994) and monocytes (Ravichandran et al., 1994) has been shown to result in the rapid formation of disulfide bonds between intra-cellular low molecular weight thiols (GSH, γ -glutamylcysteine, homocysteine and cysteine) and a range of proteins (glyceraldehyde-3-phosphate dehydrogenase, actin) in a hydrogen peroxide dependent mechanism. These disulphides were subsequently reduced back to sulphhydryls in a thioredoxin reductase dependent manner as the respiratory burst attenuated (Seres et al., 1996). As well as a potential regulatory role on protein function, S-thiolation has been proposed to play a protective role by preventing the oxidation of protein cysteine residues to irreversible oxidation products. Supportive evidence for this has been observed in the glutathiolation of the γ -glutamyl transpeptidase which appears to protect this membrane-bound enzyme from the hydrogen peroxide produced during its catabolism of GSH in the presence of metal cations (Dominici et al., 1999).

Whilst nitric oxide and many of its reactive derivatives have been implemented in the formation of mixed disulphide, probably through the oxidation of free thiols and subsequent disulphide exchange reactions, NO can also react directly with free and protein bound thiols with the formation of S-nitrosothiols (SNO). The products of these S-nitrosation reactions have been proposed to simultaneously facilitate the transport of NO *in vivo* (Kharitonov

et al., 1995), conferring NO properties on the carrier, whilst mitigating against its toxic effects (Stamler et al., 1992). A number of proteins have been identified as physiological NO reservoirs permitting the transport of this bioactive gas to effector sites *in vivo*: haemoglobin (Bonaventura et al., 2002), albumin (Tsikas et al., 2001) and calbindin D_{28K} (Tao et al., 2002). In addition to this beneficial role nitrosylation of cysteine residues has also been shown to inactivate, or indeed activate, a broad spectrum of proteins: cathepsin B (Stamler et al., 1992), ion channels (Bolotina et al., 1994; Sun et al., 2001), receptors (Lipton et al., 1993), G-proteins (Lander et al., 1995), nuclear proteins and transcription factors (Marshall and Stamler, 2002; Laval and Wink, 1994), metabolic proteins (Mohr et al., 1996). S-nitroso compounds such as S-nitroso-L-glutathione (GSNO) may inhibit enzymes responsible for the modulation of the response to oxidative stress: glutathione peroxidase (Becker et al., 1995), glutathione reductase (Becker et al., 1995) and glutathione S transferase (Clark and Sinclair, 1988). NO can be liberated from these S-nitrosoactive cysteine residues through a variety of mechanisms including metal catalysed homolytic cleavage, non-enzymatic and enzymatically catalysed transnitrosation reactions and metabolism of S-nitrosothiols by various enzymes (Hogg, 2002).

Increased concentrations of S-nitrosothiols have been reported in BAL fluid recovered from patients following lung transplantation (Gaston et al., 1993) and associated with infective pneumonia (Gaston et al., 1994). Increased concentrations have also been reported in exhaled breath condensate of patients with severe asthma, COPD, CF and in current smokers (Corradi et al., 2001). The formation of nitrosothiols depends on the availability of GSH within the RTLF, and there is some evidence that concentrations of this antioxidant are in a range of chronic pulmonary conditions (Smith et al., 1993; Cross et al., 1994), and following acute airway insults (Blomberg et al., 1999; Cantin et al., 1987). At question is whether the formation of these nitrosothiols represents an adaptive response of the airway to nitrosative stress and related to this, what the functional consequences of these elevated concentrations are.

Oxidation of tyrosine residues by ROS and RNS

During the oxidation of the aromatic amino acid tyrosine, tyrosine radicals are formed, which in the absence of repair by cellular reductants may combine to form di-tyrosines and 3,4-dihydroxyphenylalanine, generally referred to as DOPA (Davies et al., 1999). As di-tyrosine derivatives are fluorescent this property has been made use of to assess oxidative

damage to proteins in a range of model systems. Under physiological conditions the formation of these species are relatively minor but their detection has proven a reliable method of assessing protein oxidation (Huggins et al., 1993; Giulivi and Davies, 1994). Hypochlorous acid released from activated neutrophils also reacts with tyrosine residues in protein to produce 3-chlorotyrosine and 3,5-dichloro tyrosine (Kettle, 1996). Although this is a relatively minor reaction, it is the only known physiological source of chlorotyrosine. As such, chlorotyrosine may be a specific marker for oxidant activity of cells that contain MPO such as neutrophils and monocytes. Elevated chlorotyrosine concentrations have been reported in atherosclerotic lesions (Podrez et al., 2000) and lungs of patients with adult respiratory distress syndrome (ARDS), cystic fibrosis and following lung transplantation (Lamb et al., 1999; De Andrade et al., 2000; van der Vliet et al., 2000). Similarly, hypobromous acid released from eosinophils can lead to the bromination of tyrosine residues. The generation of bromotyrosine is thought to be particularly important as this product has been shown to increase 10-fold in BAL fluid obtained from asthmatic subjects following allergen challenge (Wu et al., 2000) and 100-fold in severe asthmatics admitted to emergency departments (MacPherson et al., 2001).

The most studied oxidative modification of tyrosine residues is their nitration to 3-nitrotyrosine (3-NT). This may occur through a variety of mechanisms: via the reaction of superoxide with nitric oxide to form peroxynitrate (Beckman et al., 1994) and its derivative species, by peroxidase dependent nitrite oxidations, catalysed by MPO and EPO (Eiserich et al., 1998), and through nitric oxide interactions with tyrosyl radicals (Gunther et al., 1997), and nitrous acid (Knowles et al., 1974). These mechanisms have all been demonstrated in simple *in vitro* models, however questions remain about which mechanisms operate *in vivo*, where competing reactions (including those with antioxidants) will limit the degree of protein nitration. For example the toxic action of peroxynitrite can be seriously impaired in the presence of urate (Reiter et al., 2000). Despite this proviso, there is clear evidence that nitration of proteins does occur *in vivo* (MacMillan-Crow et al., 1996; Gole et al., 2000; Viner et al., 1996; Strong et al., 1998). Elevated concentrations have been detected in animal models of hyperoxia (Haddad et al., 1994), endotoxemia (Wizemann et al., 1994), lung ischaemia/reperfusion (Ischiropoulos et al., 1995), influenza induced pneumonia (Akaike et al., 1996) and asbestosis (Tanaka et al., 1998). Nitrotyrosine concentrations have been shown to be increased in a range of pulmonary diseases: asthma (Saleh et al., 1998; Kaminsky et al., 1999) idiopathic pulmonary fibrosis (Walker et al.,

2001); Cystic fibrosis (van der Vliet et al., 1997); lung transplantation (McDermott et al., 1997); as well as in acute lung injury (Kooy et al., 1995; Haddad et al., 1994). A global increase in nitrotyrosine has been demonstrated in lung autopsy tissues from paediatric patients who had died of acute pulmonary conditions (Kooy et al., 1995), as well as in the RTLf of intensive care patients at risk of developing pneumonia and ARDS (Lamb et al., 1999; Mathy-Hartert et al., 2000). Concentrations of nitrite, nitrate and 3-NT have been shown to be elevated in BAL-fluid, cells and lung biopsy samples obtained from patients with ARDS (Gole et al., 2000; Lamb et al., 1999). Increased concentrations of 3-NT have been reported in asthma, with evidence that these levels were reduced following inhaled corticosteroid treatment (Saleh et al., 1998). Elevated concentrations of 3-chlorotyrosine, 3,3' di-tyrosines, nitrite, nitrate and 3-NT have been reported in induced sputum samples obtained from CF patients (van der Vliet et al., 2000).

Whilst the presence of 3-NT residues in protein serves as a useful marker of nitrosative stress, whether these modifications have any impact on protein function, and allied to this whether they may play an active role in tissue pathology is a critical issue. In simple model systems in which target proteins are exposed to peroxynitrite there is considerable evidence that this results in a loss of function in range of proteins: nuclear proteins (Chazotte-Aubert et al., 2000; Haqqani et al., 2002); metabolic enzymes (Souza and Radi, 1998); calcium binding proteins (Rowan et al., 2002); surfactant proteins (Zhu et al., 1996; Haddad et al., 1996; Haddad et al., 1993); tyrosine kinases (Knapp et al., 2001; MacMillan-Crow et al., 1996); phosphatases (Takakura et al., 1999), ion channels (Viner et al., 1996; Hu et al., 1994), inflammatory proteins (Sato et al., 1999; Zou et al., 1997) and antioxidant enzymes (MacMillan-Crow et al., 1996). Further, recent studies have been able to show that this loss of function is due to nitrosylation of critical tyrosine residues and not simply parallel oxidations within the protein. Of specific interest to the lung, nitration of tyrosine residues 161, 164 and 166 in SP-A have been shown to be related to its loss lipid aggregation capacity (Zhu et al., 2000). Nitration of Tyr67 on cytochrome C has been shown to have profound effects on its redox-regulating properties (Cassina et al., 2000). In studies with purified enzymes peroxynitrite has been shown to be a potent inhibitor of prostaglandin I₂ synthase, related to the nitration of a critical tyrosine residue within the active site (De Andrade et al., 2000). Cytoskeletal proteins are also thought to be significant targets for nitrosylation due to their intra-cellular abundance and high tyrosine content (Eiserich et al., 1999). Further nitration of Tyr34 allied to the formation of

3,3'-dityrosine accounts for much of the inactivation of MnSOD seen *in vivo* (MacMillan-Crow et al., 1996; Yamakura et al., 1998). This list of specific modifications is increasing, but there is still a requirement for their identification *in vivo*. Circulating levels of free 3-NT has been reported in a range of pathological conditions (Fukuyama et al., 1997 #1060) and has been shown to be taken up into A549 cells where it is irreversibly incorporated into α -tubulin through the action of tyrosine ligase, disrupting binding of microtubule-associated proteins with potential impacts on cell growth, differentiation and motility (Eiserich et al., 1999; Chang et al., 2002). Whilst these studies can demonstrate altered protein function in specific proteins in simple cell and cell free models exposed to considerable nitrosative stresses, only a limited number of studies have identified the specific proteins nitrated *in vivo* (Gole et al., 2000; MacMillan-Crow et al., 1996). In an important study by Gole et al plasma ceruloplasmin, transferrin, α 1-protease inhibitor, α 1-antichymotrypsin and fibrinogen were all shown to be nitrated with an associative loss of function in ARDs patients (Gole et al., 2000). Whilst this approach partially bridges the conceptual gap between the formation of 3-NT and altered protein function, there still remains the possibility that the parallel oxidation of other amino acids within the protein drives the effect. Therefore at present it is probably best to view these identified protein as having altered function due to general oxidation as illustrated by the presence of 3-NT. Significantly, the recent identification of a de-nitrase activity isolated from rat spleen and lung (Kamisaki et al., 1998) has raised the possibility that many of these tyrosine nitrations reactions may be reversible and hence provide a mechanism for regulating protein activity.

As mentioned previously, recent studies have demonstrated that the heme peroxidases, myeloperoxidase and eosinophil peroxidase can also catalyse tyrosine nitration from nitrite in the presence of hydrogen peroxide, providing a significant alternative pathway for their formation *in vivo* during inflammation (Eiserich et al., 1998). Increased 3-NT staining in tissue sections are often co-localised with neutrophils, and associated with elevated concentrations of MPO. Clearly the operation of this pathway is critically dependent on the concentrations of nitrite within the local environment and notably significant concentrations have been reported in the RTLf: 10–140 μ M in nasal and 10–15 μ M in alveolar compartment, increasing markedly during inflammation. The involvement of MPO in the formation of 3-NT *in vivo* has been illustrated by the observation of diminished 3-NT concentrations in the livers of MPO deficient mice compared with wild type

(Baldus et al., 2001). Recent studies have revealed that MPO released from inflammatory cells at the endothelium may undergo cellular transcytosis to the basolateral cell surface where they associate with endothelial cell glycosaminoglycans, especially fibronectin (Baldus et al., 2001). This mechanism is significant as it implies that the site of action of MPO may both be remote from its site of production and extended.

There is now good evidence that eosinophils can use nitrite as a substrate for the nitration of tyrosine residues (Wu et al., 1999). Pulmonary inflammation in asthma is characterized by the differential recruitment of eosinophils to the airway mucosa and lumen. Activated eosinophils release large amounts of cationic proteins from morphologically distinct cytoplasmic granules (Erjefalt and Persson, 2000). The most abundant of these granule proteins is a cationic heme-containing protein, eosinophil peroxidase (EPO). EPO appears to be specific for the eosinophil cell type and represents nearly 25% of the total protein mass of the secondary granule (Carlson et al., 1985). The release of EPO, together with the generation of superoxide and hydrogen peroxide from the respiratory burst (Elsner et al., 1996), leads to the generation of hypobromous acid (Slungaard and Mahoney, 1991). This potent defense mechanism can kill invading pathogens such as bacteria (Jong et al., 1980). Indeed in model systems EPO is a more potent catalyst of protein nitration than MPO under physiological conditions (Wu et al., 1999) raising the possibility that much of the nitration seen in asthma *in vivo* is related to its action. A recent study has shown a significantly higher concentration of 3-bromotyrosine in asthmatics compared with control subjects (Aldridge et al., 2002). Notably, levels of 3-chlorotyrosine were not elevated in this group emphasising the importance of EPO derived oxidants in asthma.

Protein carbonyls

There is a considerable body of evidence that the reaction of reactive oxygen and nitrogen species with lys, arg, cys, his, pro, glu, asp and thr residues in proteins leads to an increase in carbonyl groups (Stadtman and Levine, 2000). The introduction of these groups into proteins can arise through five distinct oxidative mechanisms: (1) through oxidative cleavage of polypeptide bonds by α -amidation or oxidation of glu and asp amino acid side chains; (2) by direct oxidation of lys, arg, pro and thr residues; (3) Michael addition of unsaturated aldehydes such as 4-hydroxy-2-nonenal to lys amino groups, cys sulfhydryl groups, and histidine imidazole groups; (4) by the reaction of malondialdehyde with lys to

form Schiff base derivatives; and finally (5) through the reaction of reducing sugars and their oxidation products with lys in glycation reactions. These mechanisms have been reviewed in detail by a number of authors previously (Stadtman and Levine, 2000). In contrast to many of the specific amino acid modifications already considered, where the absolute numbers of modifications are low, carbonyl modification may occur in numerous residues and may therefore have profound structural changes to a broad spectrum of proteins. A variety of highly sensitive HPLC and antibody-based techniques are available to measure protein carbonyls and thus their determination has been widely used to assess oxidative stress in a number *in vitro* and *in vivo* models. Increased concentrations of protein carbonyls have been measured following exposure of model protein solutions to ozone (Mudway et al., 1998; Berlett et al., 1996), transitional metal based free radical generating systems (Requena and Stadtman, 1999), cigarette smoke (Reznick et al., 1992) and activated neutrophils (Oliver, 1987). Increased concentrations have also been detected in the lungs of rats exposed to hyperoxia (Winter and Liehr, 1991) and paraquat (Winter and Liehr, 1991). Carbonyls concentrations are also increased in a murine model of emphysema (Yoshida et al., 2001) associated with increased NF- κ B expression and ROS formation by alveolar macrophages. Elevated protein carbonyls concentrations have also been reported in plasma from ARDS patients (Quinlan et al., 1994) and in plasma BAL fluid obtained from critically ill patients following major trauma or sepsis. Here the increased concentration of carbonyls correlated well with lipid oxidation product measurements and indices of neutrophilia and neutrophil activation (Winterbourn et al., 2000). Increased levels of protein oxidation have also been observed in tracheal aspirates from premature infants undergoing ventilation therapy, similarly related to pulmonary inflammation (Buss et al., 2000). Similar results have been shown by other groups where the increase in protein carbonyl concentrations has been shown to be positively associated with increased concentrations of matrix-metalloproteinase-9, and its inhibitor, TIMP-1 (Schock et al., 2001) suggesting that the processing of epithelial remodelling may be regulated through oxidative stress. There appears to be little evidence at present that protein carbonyl levels are elevated in asthma (Aldridge et al., 2002), although some evidence of a small increase has been reported in subjects following allergen challenge (Foreman et al., 1999) related to eosinophilia migration. Notably, in the later study, as with many of the protein oxidation markers described previously, the major protein oxidised was α 1-antitrypsin.

Further amino acid oxidative modifications

Histidine residues are converted to 2-oxohistidine (Uchida and Kawakishi, 1993) or to asparagine or aspartic acid (Berlett et al., 1996) by a variety of reactive oxygen species and oxidants. The rate of these reactions and the ultimate product formed is a function both of the oxidising species and the local amino acid environment. Whilst ozone and hydroxyl radicals randomly attack histidine residues, metal catalysed oxidation is relatively site-specific targeting residue near the metal binding site (Stadtman and Oliver, 1991). Phenylalanine can also be oxidised to a range of products: ortho- and metaptyrosine (Huggins et al., 1996), 2-, 3-, 4-hydroxy and 2,3-dihydroxy derivatives (Gieseg et al., 1993; Dean et al., 1993). Protein-cross-linking in response to oxidation results in the formation of larger molecular weight species. These aggregates are formed through a variety of mechanisms: the formation of disulphide bridges, through covalent C–C bond formation arising from the interaction between two carbon-centred radicals, by interactions between lysine residues on separate proteins with lipid peroxidation products, or finally, via formation of a Schiff base cross link between a carbonyl group on one protein and a N-amino group of a lysine residue on a second. None of these modifications have been addressed in detail in models of pulmonary injury to date.

Recently considerable attention has focused on the formation of protein lipid oxidation product adducts, specifically with the lipid peroxide degradation products 4-hydroxynonenal and malondialdehyde in a number of diseases characterised by oxidative stress (Eaton et al., 2001; Walker et al., 2001; Houghlum et al., 1990). The focus on this protein oxidative modification has been driven by the availability of commercially available antibodies against 4-HNE-protein adducts, permitting identification and quantification of modified proteins by Western blotting and immunohistochemistry (Eaton et al., 2001). In addition, elevated concentrations of these lipid oxidation species have been identified in almost all of the major pulmonary diseases characterised by acute and chronic inflammation making the formation of these adducts highly feasible *in vivo*. Increased concentrations of these adducts have been reported in the lungs of smokers with and without COPD (Rahman et al., 2002). Notably, 4-HNE concentrations in the pulmonary epithelium, airway endothelium and particularly in neutrophils of the COPD patients were found to be inversely associated with lung function. Significantly, the formation of these protein-lipid adducts have been shown to have profound effects on protein function. For example the binding of lipid

oxidation products to the epidermal growth factors receptor has been shown to result in the phosphorylation of the receptors, with subsequent stimulation of the ERK pathway (Liu et al., 1999).

Summary

The lung is assaulted on a daily basis by a range of toxic insults. Whether these are infectious agents, allergens, or air pollutants they act upon the lung by eliciting oxidation reactions, either directly, or through inflammatory processes. Measurement of protein oxidation products therefore permits the degree of oxidative stress to be assessed and indicates that endogenous antioxidant defences are overwhelmed. The range of protein oxidation products observed is diverse and the nature and extent of specific oxidation products may inform us about the nature of the damaging ROS and NOS. Recently, there has been a significant shift away from the measurement of these oxidation products simply to establish the presence of oxidative stress, to a focus on identifying specific proteins sensitive to oxidation and establishing the functional consequences of these modifications. In addition the identification of specific enzyme systems to repair these oxidative modifications has led to the belief that protein function may be regulated through these oxidation reactions.

Future work

A major challenge for researchers working in this area will be to try and establish which specific amino acid oxidations are related to altered protein function. Oxidised proteins are likely to carry a range of oxidative modifications and thus determining which are the crucial changes will require careful work. In this respect it is clear that some protein modifications occur at a low frequency, i.e., try and met oxidations, while others have a much more global effect, e.g. carbonyl formation. Determining which, if any, of these modifications are most important *in vivo* is a clear challenge. Much more work is required to clarify specific oxidation products that arise due to the wide range of ROS and RNS that are present under defined conditions. Likewise, the interaction between endogenous antioxidant defences and protein oxidation requires more study. It is likely that certain amino acids are oxidised on proteins to spare other critical residues from oxidation, i.e. they are performing an antioxidant role. Much of this work will most easily be approached with *in vitro* studies. However, clearly it will be very important

to test the relevance of these data within the *in vivo* situation.

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