Topical Reviews

Characterization of N-acetylcysteine and ambroxol in anti-oxidant therapy

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Reactive free oxygen radicals are known to play an important role in the pathogenesis of various lung diseases such as idiopathic pulmonary fibrosis (IPF), adult respiratory distress syndrome (ARDS) or cystic fibrosis (CF). They can originate from endogenous processes or can be part of exogenous exposures (e.g. ozone, cigarette smoke, asbestos fibres). Consequently, therapeutic enhancement of anti-oxidant defence mechanisms in these lung disorders seems a rational approach. In this regard, N-acetyl-L-cysteine (NAC) and ambroxol have both been frequently investigated.

Because of its SH group, NAC scavenges H₂O₂ (hydrogen peroxide), -OH (hydroxyl radical), and HOCI (hypochlorous acid). Furthermore, NAC can easily be deacetylated to cysteine, an important precursor of cellular glutathione synthesis, and thus stimulate the cellular glutathione system. This is most evident in pulmonary diseases characterized by low glutathione levels and high oxidant production by inflammatory cells (e.g. in IPF and ARDS).

NAC is an effective drug in the treatment of paracetamol intoxication and may even be protective against side-effects of mutagenic agents. In addition NAC reduces cellular production of pro-inflammatory mediators (e.g. TNF-α, IL-1).

Also, ambroxol (trans-4-(2-amino-3,5-dibromobenzylamino)-cyclohexane hydrochloride) scavenges oxidants (e.g. -OH, HOCI). Moreover, ambroxol reduces bronchial hyperreactivity, and it is known to stimulate cellular surfactant production. In addition, ambroxol has anti-inflammatory properties owing to its inhibitory effect on the production of cellular cytokines and arachidonic acid metabolites. For both substances effective anti-oxidant and anti-inflammatory function has been validated when used in micromolar concentrations. These levels are attainable in vivo in humans. This paper gives an up-to-date overview about the current knowledge of the hypothesis that oxidant-induced cellular damage underlies the pathogenesis of many human pulmonary diseases, and it discusses the feasibility of anti-oxidant augmentation therapy to the lung by using NAC or ambroxol.

Introduction

Reactive oxygen species (ROS) are constantly formed in the lung and serve a physiological role. Once radicals are formed they can (a) destroy micro-organisms, normal or neoplastic mammalian cells and (b) modulate the inflammatory response (e.g. mediator release of inflammatory cells). However, when generated in excess, or in inappropriate environments, and when anti-oxidant defence is insufficient, radicals can damage DNA, lipids, proteins and carbohydrates [for review see 1–3]. In the lung in particular, various diseases are known where oxygen metabolites play a predominant role in the pathogenesis (Table 1).

Based on this knowledge, anti-oxidant augmentation therapy seems to be a rational approach in pulmonary diseases characterized by an overload of radicals or lack of anti-oxidants. In this regard, N-acetyl-L-cysteine (NAC) and ambroxol, both widely used in Europe as mucolytic agents, have been investigated thoroughly in the recent past. The purpose of this article is to summarize these findings in order to assess clinical feasibility of anti-oxidant therapy with these drugs.

The Role of Oxidants in Living Systems

FREE RADICALS

A free radical is any species capable of independent existence that contains one or more unpaired electrons, where an unpaired electron is one that occupies an atomic or
Reactive oxygen intermediates are regarded to play a considerable role (a) in the pathogenesis of various pulmonary inflammatory disorders and (b) after inhalation of various substances [summarized from (59,155-158)]

<table>
<thead>
<tr>
<th>Disease or exposure</th>
<th>Source of oxidant</th>
</tr>
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<tbody>
<tr>
<td>Adult respiratory distress syndrome (ARDS)</td>
<td>Stimulated PMN cells</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>Stimulated PMN cells, lack of fat-soluble anti-oxidants (caused by exocrine pancreatic insufficiency)</td>
</tr>
<tr>
<td>Idiopathic pulmonary fibrosis (IPF)</td>
<td>Stimulated PMN cells, and alveolar macrophages, pulmonary GSH deficiency</td>
</tr>
<tr>
<td>Asthma</td>
<td>Stimulated PMN cells and eosinophils</td>
</tr>
<tr>
<td>Hyperoxia</td>
<td>O$_2$– formation from transport chains</td>
</tr>
<tr>
<td>Ozone</td>
<td>O$_2$: formation of O$_2$–, -OH and H$_2$O$_2$, release of cyclo-oxygenase metabolites of arachidonic acid</td>
</tr>
<tr>
<td>Asbestosis</td>
<td>Fe$^{2+}$, stimulated macrophages</td>
</tr>
<tr>
<td>Silicosis</td>
<td>Fe$^{2+}$ after formation of silicato-iron complexes</td>
</tr>
<tr>
<td>Paraquat toxicity</td>
<td>Redox cycling</td>
</tr>
<tr>
<td>Bleomycin toxicity</td>
<td>Formation of O$_2$– and -OH from Fe$^{2+}$-bleomycin complex</td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td>H$_2$O$_2$, NO, peroxyl compounds, quinones, stimulated PMN cells and macrophages</td>
</tr>
<tr>
<td>Ionizing radiation</td>
<td>O$_2$– formation by ionization</td>
</tr>
<tr>
<td>HIV infection</td>
<td>Systemic deficiency of acid-soluble thiols</td>
</tr>
</tbody>
</table>

### Table 2. Examples of oxygen species [the terms oxidants and ROS includes oxygen radicals and other derivatives of O$_2$ (non-radicals) (4)]

<table>
<thead>
<tr>
<th>Formula</th>
<th>Description</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>O$_2$–</td>
<td>Superoxide anion</td>
<td>Produced by a number of enzymes, auto-oxidation reactions and non-enzymatic electron transfers. Conversion to H$_2$O$_2$ [by superoxide dismutase (SOD) and O$_2$] or -OH (Fenton reaction).</td>
</tr>
<tr>
<td>-OH</td>
<td>Hydroxyl radical</td>
<td>Highly reactive Occur as organic radicals during the breakdown of peroxides of lipids (peroxidation)</td>
</tr>
<tr>
<td>RO$_2$, RO'</td>
<td>Peroxyl, alkoxyl radicals</td>
<td>Occur as organic radicals during the breakdown of peroxides of lipids (peroxidation)</td>
</tr>
<tr>
<td>NO, NO$_2$</td>
<td>Oxides of nitrogen</td>
<td>Nitric oxide and nitrogen dioxide can be found in vivo (NO) and in polluted air (NO$_2$).</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
<td>H$_2$O$_2$ occurs from O$_2$– by many enzymatic reactions. From H$_2$O$_2$, -OH can be formed.</td>
</tr>
<tr>
<td>HOCl</td>
<td>Hypochlorous acid</td>
<td>It is formed by action of myeloperoxidase in inflammatory reactions Formed in the environment.</td>
</tr>
<tr>
<td>O$_3$</td>
<td>Ozone</td>
<td>Formed in the environment.</td>
</tr>
</tbody>
</table>

Molecular orbital by itself (4). ROS is a collective term that includes not only free oxygen radicals but also some derivatives of O$_2$ that do not contain unpaired electrons such as H$_2$O$_2$ (hydrogen peroxide), $^1$O$_2$ (singlet oxygen), O$_3$ (ozone) and HOCl (hypochlorous acid) (Table 2). The reactivity of the most frequently occurring ROS is as follows (5):

$$O_2<^1O_2<HOCl<HOCl$$

Oxidants are generated by various chemical processes (e.g. certain drugs, metals) and enzymatic reactions, or they can directly be inhaled (e.g. smoking, ozone, hyperoxia) [for review see (6–8)].

Myeloperoxidase, present in polymorphonuclear (PMN) cells and released during respiratory burst, converts H$_2$O$_2$ into HOCl, another highly toxic oxidant which can further react to form more long-lived chloramines (9). Triggered by SOD, O$_2$– (superoxide anion) quickly dismutates to H$_2$O$_2$ from which highly reactive -OH (hydroxyl radical) can be formed in the presence of transition metals such as iron. Iron- (or copper-) catalysed reactions are often referred to as either the Haber-Weiss reaction (formation of -OH from H$_2$O$_2$ and O$_2$–) or
the Fenton reaction (formation of \( \cdot \text{OH} \) from \( \text{H}_2\text{O}_2 \)) (10,11). Since \( \text{H}_2\text{O}_2 \) easily diffuses through cell membranes, \( \cdot \text{OH} \) formation occurs extra- as well as intracellularly. \( \cdot \text{OH} \) always causes site-directed cell damage where it occurs.

Generation of oxygen radicals by inflammatory cells is known to play an important part in the killing of several bacterial and fungal strains. \( \text{H}_2\text{O}_2 \) is used in a thyroid peroxidase mediated reaction to produce thyroid hormones or it can induce gene expression (e.g. cytoplasmatic gene transcription factor) (5,12). Because tumour necrosis factor (TNF), hyperthermia, and anti-cancer drugs such as Adriamycin produce oxidants that cause tumour cell lysis, ROS seem to be meaningful in the natural defence against the development of cancer (13). Apart from the natural role oxygen radicals play in the host defence system, in inflammatory processes they notably can contribute to the severity of various pulmonary diseases. Since oxygen radicals are able to induce DNA-strand breaks, which activate oncogenes such as \text{K-ras}, \text{C-Raf-1} and \text{c-fos}, oxygen radicals are also associated with an increased incidence of malignancies as proposed in asbestos exposure related tumours (14,15).

Activated leukocytes (both PMN cells and macrophages) release highly reactive reduced oxygen species such as \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \) and others (\( \cdot \text{OH}, \text{HOCl}, \text{NO}_2 \), and \( \text{HO}_2 \)). The local concentrations of oxidants formed by PMN cells, which occur during normal cellular oxidative phosphorylation, can be extremely high: \( 2 \times 10^9 \) PMN cells stimulated with \( 10^{-6}\text{M F-Met-Leu-Phe} \) produce \( 10^{-4}\text{M O}_2^- \) within 1 min (9). Secretion of toxic oxygen metabolites into the extracellular milieu is known as respiratory burst.

Also, inhalation of inorganic as well as organic particles generates oxidant stress via activation of inflammatory cells. Asbestos fibres (e.g. crocidolite) have a high redox potential through their iron content (up to 28%), forming \( \cdot \text{OH} \) in the Fenton reaction (16). Asbestos fibres and silica in addition trigger cytokines (e.g. TNF) release which stimulates inflammatory cells to produce large amounts of oxidants. In rat lungs inhaled crocidolite enhances antioxidative activity, and alveolar macrophages have been seen to undergo lipid peroxidation that can be blocked by anti-oxidants (17,18).

Interestingly, various cellular mediators released by stimulated inflammatory cells may further contribute to the oxidant load. Inflammatory mediators such as TNF, IL-1, IL-6, IL-8, various lipid mediators such as arachidonic acid metabolites prostaglandins, leukotrienes (e.g. \( \text{B}_4 \)), the phospholipid platelet-activating factor and others are involved in the autocrine or paracrine activation of macrophages, neutrophils, fibroblasts, epithelial cells and endothelial cells (19–22). Both activated blood monocytes and neutrophils are attracted into the alveolar space by eicosanoids and cytokines released from these alveolar macrophages and thus enhance oxidant levels in the epithelial lining. Arachidonic acid metabolites can initiate oxidant release of activated inflammatory cells and enhance anti-oxidant enzyme expression in bronchoepithelial cells (23–27). Phospholipase \( \text{A}_2 \) releases arachidonic acid, which is involved directly or as a co-factor in the activation of the neutrophil respiratory burst (1,28). In contrast, mediators such as TNF, lipopolysaccharide (LPS) and IL-1 induce in epithelial cell types expression of anti-oxidants (e.g. MnSOD) (26,29–31).

THE PATHOGENESIS OF OXIDANT RELATED INJURY

The term lung injury incorporates a wide range of phenomena characterized by widely differing manifestations, including, among others, functional alterations in gas exchange, excess of lung water, alterations of pulmonary haemodynamics, increased vascular permeability and histological evidence of structural damage. An impressive variety of insults may cause acute and chronic lung injury. One group of these insults affects the lung parenchyma directly, whereas others affect the lung via systemic mechanisms (32).

Lung injury is clearly marked by measurable changes as result of an inflammatory response where circulating PMN cells accumulate in the local vasculature and migrate through the vessel wall, the interstitium, and the epithelial barriers to gain access to the alveolar lumen.

Hyperoxia leads to a significant increase of intracellular \( \text{O}_2^- \) formation which may be one of the reasons why patients ventilated under hyperoxic conditions more easily develop ARDS (33). Recent experiments done in healthy volunteers exposed to 100% \( \text{O}_2 \) for 12–18 h by a full-face mask not only demonstrated that oxygen caused tracheobronchitis in most of the volunteers but also revealed that epithelial cells are not able to protect themselves from free radicals by triggering anti-oxidant (SOD and catalase) gene expression during this time period (34). Thus, oxidant-related cell injuries such as tracheobronchitis or ARDS in humans or low survival rates in animal experiments are typical consequences (33,35).

Bleomycin and the herbicide paraquat cause oxidant formation as a result of redox cycling which is Fe dependent (36,37). Because of its high affinity to DNA, bleomycin induces site-directed \( \cdot \text{OH} \) formation which causes crucial DNA damage. Certain xenobiotics, activated by intracellular reductases and thereby undergoing oxidative redox cycling, exert cell damage via intracellular generation of \( \text{O}_2^- \). Lung injuries caused by paraquat, bleomycin and nitrofurantoin are considered a chemical form of oxygen toxicity. As a consequence paraquat toxicity, characterized by alveolitis, oedema and bleomycin, is used as a model for acute lung injury and pulmonary fibrosis (1,38).

Cigarette smoke, which has been estimated to contain \( 10^{14} \) oxidants per puff, and ionizing radiation induce inflammatory cells to release oxidants, but both may also cause cell injury through direct oxidant formation (39,40). Ozone, which contains unpaired electrons, degrades to highly reactive \( \cdot \text{OH} \).

Oxidants indirectly contribute to pulmonary injury by inactivating anti-proteolytic proteins such as \( \alpha_1 \)-antitrypsin (\( \alpha_1 \)-AT) and secretory leukoprotease inhibitor (SLPI). ROS cause methionine sulphoxide formation at the active site and thus inactivate these anti-proteases. High methionine
sulphoxide levels in bronchoalveolar lavage (BAL) fluid of patients with ARDS and in BAL fluid of cigarette smokers indicate that oxidative processes inactivate proteases in their airways more frequently than in normal volunteers (41). Because α1-AT is the most important protease to inactivate neutrophil elastase specifically, part of the oxidant-induced injury may also be due to secondary proteolytic damage caused by inactivation of α1-AT. Malfunction of α1-AT and therefore high activity levels of elastases are known to promote the development of lung emphysema (41). Like anti-proteolytic agents, anti-oxidants such as SOD can also be inactivated by oxidants (e.g. H₂O₂) (1,42).

There is multiple evidence that oxidative events associated with acute lung injury may also induce lipid peroxidation and consequently affect the lung surfactant system (43). Disturbance of the surfactant systems leads to acceleration of further lung injury (44).

Anti-oxidants

PRINCIPLES OF ANTI-OXIDANT DEFENCE

Normally, in the lung exists an oxygen-rich environment sensitively balanced between the toxicity of oxidants and protective measures of various intracellular and extra cellular anti-oxidants. Overlapping activities of anti-oxidants (e.g. glutathione and catalase are both scavenging H₂O₂) indicates tight control of redox balance specifically to protect the cells without blocking the physiological role of cellular oxidants. To encounter the damaging effect of oxygen metabolites, the cells in the lung have a sophisticated network of anti-oxidant defence mechanisms. If exposed to oxidants, pulmonary cells (e.g. bronchoepithelial cells) are even able to enhance their anti-oxidant defence. For example, in smokers glutathione levels in BAL fluid are generally higher than in non-smokers; asbestos fibres cause an increase in MnSOD mRNA levels (45–48). However, the interrelationship between ROS and anti-oxidants in humans is very complex, and thus just the basic mechanism should be mentioned here [for further reviews see (7,49–51)].

Oxidant-scavenging systems can be categorized into the following groups: (a) enzyme systems, (b) fat-soluble compounds, (c) water-soluble compounds and (d) high molecular weight anti-oxidants (49). According to this classification, these agents are present in varying degrees in the intracellular or extracellular spaces: (a) SOD, catalase, glutathione redox cycle compounds and glutathione peroxidase, glutathione reductase, glucose-6-phosphodi-hydrogenase; (b) vitamin E, β-carotene, bilirubin; (c) vitamin C, uric acid, glucose, cysteine, cystamine, reduced glutathione (GSH), taurine; (d) tracheobronchial mucus, albumin. SOD, catalase and the compounds of the glutathione redox cycle are the primary intercellular anti-oxidant defence mechanism to cope with ROS.

The most important H₂O₂-removing system in human cells is the glutathione redox cycle including the glutathione peroxidases (GSHPX). GSH is oxidized to GSSG and thus removes H₂O₂. Glutathione reductase regenerates GSH from GSSG, with NADPH as a source of reducing power (52).

from GSSG, with NADPH as a source of reducing power (Fig. 1) (52). Located in the peroxisomes, catalase catalyses like GSH the dismutation of H₂O₂ into water and O₂:

\[ 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \]

Because of their conversion into ·OH and into reactive radical metal complexes, the conversion of O₂⁻ and H₂O₂ into less toxic products is from major importance in living cells (53). Therefore, sufficient anti-oxidant augmentation therapy can only be expected when these oxidants are scavenged or cellular metabolism leading to increased production of oxidants is attenuated.

PHARMACOLOGICAL INTERVENTIONS TO ENHANCE CELLULAR ANTI-OXIDANT DEFENCE

SOD, catalase and GSH in particular, but also a whole variety of other substances with direct and indirect anti-oxidant capabilities (see previous section), prevent oxidative cell injury in cell culture as well as in vivo in animal models and in humans (38,49,54–56). However, the effectiveness of anti-oxidant treatment in vivo faces a dilemma: the administration of SOD (although available in recombinant form) and catalase is limited by the large molecular mass of both proteins. Liposome encapsulation (or conjugations with polyethylene glycol) has been successfully used to enhance SOD, catalase and GSH efficiency, but absence of phagocytes was required (57,58). Acrosolization of pure GSH was characterized by a low half-life (55). Vitamin E protects just vitamin-E-deficient animals. Although vitamin C can scavenge oxygen species and thus reduce the occurrence of malignancies, it is generally not considered as a major anti-oxidant because it has additional pro-oxidant
properties (38,49). Another approach would be to stimulate cells to produce anti-oxidants. NAC has such a potential, because when deacetylated it provides cysteine for cellular glutathione synthesis (49,59). Other substances such as ambroxol also have direct anti-oxidant properties and like NAC a whole variety of indirect anti-oxidant functions. Ambroxol is able to reduce oxidant-related cell damage through inhibition of phospholipases, stimulation of the lung surfactant system (e.g. stimulation of alveolar phospholipid secretion), inhibition of cytokines such as IL-1 and TNF, and inhibition of chemotaxis response of neutrophils to formyl-methionyl-leucyl-phenylalanine (FMLP) (60-62).

**N-acetylcysteine**

**DIRECT ANTI-OXIDANT ACTION OF N-ACETYLCYSTEINE**

NAC, which was developed in the 1960s, is the N-acetyl derivative of the naturally occurring amino acid L-cysteine (63). Because of its ability to reduce disulphide bonds it is widely used to reduce viscosity and elasticity of mucus and is virtually non-toxic (64). The molecular weight is 163.2 g mol⁻¹ and the molecular formula is C₇H₁₅NO₃S. The oxidant scavenger function of NAC can easily be explained by the chemistry of this molecule. Because of its SH group [Fig. 2(a)] NAC has the potential to interact directly with oxidants such as H₂O₂ to form H₂O and O₂ (64,65). In this reaction two NAC molecules are oxidized to a disulfide bond [Fig. 2(a)]. The rate constant with O₂⁻ is significantly low (64,66). NAC, like many thiols such as GSH, is an excellent scavenger of ·OH radical (rate constant: 1.36 x 10¹⁵ M⁻¹ s⁻¹). In addition, NAC also scavenges HOCI (64).

**GLUTATHIONE PRECURSOR FUNCTION OF N-ACETYLCYSTEINE IN VITRO AND IN ANIMAL EXPERIMENTS**

In addition to oxidant scavenger function, there is plenty of evidence showing that NAC promotes cellular glutathione production, and thus NAC reduced or even prevented oxidant mediated damage to cell culture or animals. Administration of ≥10⁻⁵ M NAC to A549 cells (ATCC), a human derived cell line with epithelial characteristics, resulted in an approximately ten-fold increase of intracellular glutathione levels (65). Because bovine pulmonary artery endothelial cells and lung type II epithelial cells transported cysteine more efficiently than cystine it seems likely that cysteine is the principal substrate for cellular glutathione production (67). Cysteine is derived from N-deacetylation processes of NAC and/or from the reduction of cystine to cysteine (68). Although additional mechanisms have been discussed, it seems likely that N-deacetylation of NAC represents the key mechanism for the cellular glutathione precursor effect (67,69,70).

Wagner et al. investigated in 12 dogs the preventive effect of intravenous NAC against O₂ toxicity (71). Concomitantly, NAC treatment resulted in a significant decrease of pulmonary vascular resistance, and arterial carbon dioxide tension, delayed development of abnormal ventilation-perfusion relationships and reduced alveolar and interstitial oedema (71). In endotoxin-exposed rats NAC treatment improved ARDS-like injury by ameliorating structural lung damage and reducing lung wet weight and lung albumin leakage and it resulted in a reduction of thromboxane B₂ and 6-keto-PGF₁α (prostaglandin) in BAL fluid. The authors related these results mainly to the GSH precursor effect of NAC (72).

NAC also protects against pulmonary O₂ toxicity as well as against ischaemic and reperfusion damage. Various physiological evaluation measures in dogs ventilated with 100% O₂ for 54 h were improved in the NAC group (71). When only given on reperfusion after artificial ischaemia in isolated rabbit hearts, NAC had no altering effect on cellular glutathione levels. However, when NAC was administered prior to coronary ischaemia (60 min), tissue GSH was increased by almost 40%, and ischaemia-induced decrease of GSH and protein SH, as well as leakage of creatine kinase, was limited. Interestingly, mitochondria, isolated after ischaemia, maintained their natural oxidative phosphorylating capacities (73).

In addition to this evidence of its anti-oxidant functions, NAC has also anti-inflammatory capabilities. Oral administration of NAC reduces humoral markers in BAL
NAC, NAL revealed similar H₂O₂ scavenger capabilities as well as cellular GSH precursor function when used in micromolar concentrations, which was recently inhibitory functions on free radicals and whether it has demonstrated low levels in alveolar macrophages of smokers (75). Mucolytic activity of NAL may be more potent than the zation without causing significant side-effects (76). The tion NAC is quickly absorbed and undergoes rapid and extensive metabolism in gut wall and liver, resulting in a half-life of total acetylcysteine is 5.6 h (82). At lo-12 h after oral administration of various doses of NAC had no signifi-cant effect on glutathione concentrations in BAL lining or in bronchoepithelial wall in traces at best. However, NAC frequently has to be used to balance the oxidant–anti-oxidant system in human pulmonary diseases.

**ANTI-OXIDANT PROPERTIES OF N-ACETYLCYSTEINE IN HUMANS**

To investigate anti-oxidant properties of NAC, in most studies doses between 200 mg and 600 mg daily (oral administration), and up to 150 mg kg⁻¹ (i.v. as a loading dose) in patients with ARDS, were administered (75,87-90). Overall, it is difficult to relate successful treatment exclusively to the anti-oxidant effect of NAC since it also has mucolytic properties. Most studies indicate that the therapeutic potential of NAC in the treatment of inflammatory disorders of the human lung is associated with its direct scavenger effect and/or its ability to increase cysteine, the main cellular glutathione precursor. Some authors, however, failed either to increase anti-oxidant function in human lung or to reduce the activation state of inflammatory cells by oral NAC administration (91-93).

**NAC in Healthy Individuals**

The effect of NAC in enhancing anti-oxidant defence in the lungs of healthy volunteers is marginal. In 11 normal, non-smoking volunteers (aged 24–37 years) O₂⁻ as well as H₂O₂ release of freshly isolated neutrophils from whole blood remained unchanged after oral administration of 600 mg NAC daily for 5 days (93). In normal individuals i.v. administration of various doses of NAC had no significant effect on glutathione concentrations in BAL fluid. Compared with baseline values (9.79 ± 0.17 μM),...
administration of 600 mg NAC (0.92 ± 0.33 μM), 1.8 g NAC (1.4 ± 0.4 μM) and 4.8 g NAC (1.33 ± 0.46 μM) did not significantly increase total glutathione levels (3 h after i.v. application) (94). Meyer et al. hypothesized that lack of glutathione increase in BAL fluid after NAC administration might be due to various reasons. First, NAC may support glutathione synthesis only in situations of increased demand on the cellular glutathione pool following oxidative stress (e.g. after paracetamol intoxication or in patients suffering from IPF) (94). Second, the rapid clearance of most of the NAC and its products could limit its effect and, third, increasing glutathione levels in BAL fluid might suppress local glutathione synthesis by feed-back inhibition (94,95).

In addition to the failure of NAC to increase glutathione or cysteine levels in BAL fluid (or ELF) in healthy individuals, NAC (200 mg t.i.d.) significantly (P<0.001) reduced various inflammatory parameters in BAL fluid in 11 healthy smokers: lactoferrin, eosinophil cationic protein, anti-chymotrypsin and chemotactic activity of neutrophils. In serum—plasma the concentrations of myeloperoxidase and elastase were also reduced by NAC (74).

Kleinfeld et al. (92) found in six healthy normolipidaemic adult volunteers that NAC (2 × 600 mg for 4 weeks, followed by 2 × 1200 mg for 2 weeks) had no effect on the susceptibility of LDL (low-density lipoprotein) oxidizability. Unexpectedly and in contrast to other investigations, NAC administration even resulted in reduction of plasma susceptibility of LDL (low-density lipoprotein) oxidizability. Among others, patients with ARDS, IPF or COPD have been the primary targets for clinical studies to evaluate the efficacy of NAC in anti-oxidant therapy. ARDS is of multifactorial origin, and at a certain stage of its development strong oxidative stress through massive PMN activation becomes a predominant condition. Concomitant glutathione-mediated anti-oxidant protection is low in the alveolar fluid of these patients (96). Mechanical ventilation with high concentrations of oxygen further enforces the release of reactive oxygen metabolites (33,97). To balance the oxidant-anti-oxidant system, patients with ARDS (n=30) were treated i.v. with high doses of NAC. After an initial dose of 150 mg kg⁻¹ followed by 24 mg kg⁻¹ every 4 h, initially low plasma and red cell glutathione levels increases. In addition, improvements of chest radiograph oedema, vascular resistance, oxygen delivery and oxygen consumption were achieved (98). Spies et al. (89) confirmed this result in a placebo-controlled study; in the NAC-responder group (50% of all patients) 65% of patients with a septic shock who initially received 150 mg kg⁻¹ in 15 min of NAC, followed by 12.5 mg kg⁻¹ h⁻¹, had a better survival rate (89). With i.v. NAC (40 mg kg⁻¹ day⁻¹) Suter and coworkers improved systemic oxygenation and reduced the need for ventilatory support in patients presenting with mild-to-moderate acute lung injury. However, the development of ARDS and patients' survival (mortality) was not significantly reduced by this therapy; the 1 month mortality rate was 22% for the NAC group (n=32) and 35% for the placebo group (n=29) (99).

IPF is characterized by alveolar inflammation, exaggerated release of reactive oxygen metabolites and subnormal concentrations of glutathione in the ELF (100,101). Direct glutathione administration via the aerosol route but also NAC given i.v. or by mouth increased significantly BAL fluid (or ELF) glutathione levels (55,90,94). In 17 non-smoking patients with biopsy-proven IPF receiving 3 x 600 mg NAC day⁻¹ for 5 days BAL fluid total glutathione levels increased significantly (P<0.006) from 0.99 ± 0.12 to 1.54 ± 0.24 μM (90). Also, i.v. administration of different NAC concentrations to patients with IPF increased total glutathione in BAL fluid significantly (P<0.03). BAL fluid total glutathione levels rose from 0.99 ± 0.25 μM to 1.79 ± 0.37 μM (after 1.8 g NAC). A further increase of BAL fluid total glutathione levels after higher doses (4.8 g NAC) was not observed (1.47 ± 0.34 μM) (94). High concentrations of total glutathione were almost entirely due to GSH (about 94–97%)(94). However, these results do not mandate clinical efficacy of NAC treatment because just biochemical alterations have been demonstrated in these studies. This has still to be demonstrated in long-term studies.

Also, in COPD patients NAC treatment was tried to improve anti-oxidant levels although this disease, with the possible exception of severe cases, is not characterized by glutathione deficiency. NAC has been used for over two decades in the treatment of COPD and some studies demonstrated beneficial effect, but others have not (102–104). Oral administration of NAC to patients with COPD (two groups, one received 600 mg once daily, the second 600 mg thrice daily) resulted in a significant (P<0.05) increase of NAC in blood 1.5 h after the drug was given (after 5 days treatment). Sustained elevation of plasma glutathione and cysteine levels was only observed in the group treated with the higher dosage. In contrast, neither GSH nor cysteine levels rose in BAL fluid, ELF or lung tissue (87).

These results, a negative effect in healthy volunteers or patients with COPD (normal glutathione levels in BAL fluid) and beneficial effect of NAC treatment in patients with IPF or ARDS (low glutathione levels in BAL fluid, high oxidant load), support the hypothesis of negative feedback inhibition (see previous section) (95). In agreement with this concept, patients with a hereditary deficiency of glucose-6-phosphodehydrogenase, who are more sensitive to oxidative stress, were also successfully treated with NAC. In these patients NAC treatment augmented not only glucose-6-phosphodehydrogenase activity but also reduced effective lipid peroxidation and made these erythrocytes more resistant to incubation with acetylphenylhydracine, an agent causing oxidative stress (105,106).

NAC is virtually non-toxic. When used in humans NAC rarely causes anaphylaxis, tachycardia or hypotension (107,108). Only very high concentrations in in vivo and in vitro experiments revealed that >10⁻³ M reduces chemotaxis and >0.1 M increases cytotoxicity of PMN cells (107). Because of the low bioavailability, it is not feasible to achieve these concentrations in humans (84,109).
N-ACETYL-CYSTEINE AS AN ANTIDOTE

From 5% to 10% of paracetamol is metabolized to N-acetylbenzochionimine. This metabolite reacts with thiols and thus can be detoxified. However, toxic amounts of paracetamol (intake about ≥6 g) overwhelm cellular glutathione supplies, eventually causing liver necrosis (110). Because approximately 70% of NAC is metabolized in the liver, NAC has been proven to be a useful antidote in paracetamol intoxication, which conjugates with reduced glutathione (63,111). In the liver, such antidote action is related to NAC as an effective precursor to supply intracellular cysteine, which is intracellularly transformed into GSH. Thus, the NAC mediated GSH increase not only conjugates paracetamol to less toxic metabolites but it also makes GSH available for anti-oxidant defence purposes intracellularly as well as extracellularly (63).

NAC given orally also has a cardioprotective effect in patients treated with doxorubicin, it reduces cyclophosphamide-induced haemorrhagic cystitis and it attenuates bieomyicin-related changes in lung function (112,113).

Patients successfully treated for lung cancer remain at high risk to develop a secondary primary tumour. In Euroscan [the European Chemoprevention Study under the auspices of the European Organization for Research and Treatment of Cancer (EORTC)] patients after successful treatment of lung or head and neck cancer are randomized as follows: NAC (600 mg day⁻¹ for 2 yr), retinol palmitate, the combination of both or no drugs. To date, more than 2600 patients have been included. Only 8% suffered from side-effects (mild gastrointestinal symptoms) (114,115).

With NAC, the authors hope to reduce the risk of second cancers. Reduction of the development of second tumors is based on the knowledge that thiols inhibit the mutagenic response to potent carcinogens such as benzo[a]pyrene, 2-aminofluorene and aflatoxin β1, reduces oxidant-mediated DNA damage and stimulates DNA repair mechanisms (115).

**Ambroxol**

**PHYSIOLOGICAL EFFECTS OF AMBROXOL**

Ambroxol [trans-4-(2)-amino-3,5-dibromobenzylamino)cyclohexane hydrochloride; Fig. 2(b)] stimulates synthesis and secretion of surfactant by type II pneumocytes (116,117) and inhibits sodium absorption by airway epithelial cells (118). It can also accelerate the maturation of the foetal lungs by increasing the lecithin: sphingomyelin ratio (119). Ambroxol is used as a mucolytic drug and lower sputum viscosity by normalizing secretions from bronchial glands. In this context, ambroxol, has been widely tested to prevent chronic bronchitis exacerbations, to improve the clearance mechanism of the epithelial surface of the airways, to decrease bronchial hyperreactivity and even to promote secretion mechanisms in patients with otitis media and to reduce the occurrence of neonatal respiratory distress syndrome by induction of foetal lung maturation (120–125).

**ANTI-OXIDANT ACTIVITY OF AMBROXOL IN VITRO**

At concentrations of 10⁻⁶→10⁻⁵ M and 2.5–7 × 10⁻⁵ M respectively ambroxol revealed a capacity to inhibit -OH- and HOCl-mediated reactions (126). As a scavenger of these two ROS ambroxol was almost equipotent to dimethylsulphoxide and methionine (126). The inhibition of O₂⁻-dependent auto-oxidant of pyrogallol and decomposition of H₂O₂ by ambroxol was low over a large concentration range. The capacity of ambroxol to scavenge HOCl and -OH can explain the above-mentioned inhibitory effect of this drug on chloramine-T- and cigarette-smoke-induced decrease of serum elastase inhibitory capacity (127,128). Both of these factors inactivate α₁-AT via generation of HOCl and -OH.

Ambroxol was shown to inhibit lipid peroxidation in liver homogenates exposed to the Fe²⁺-ascorbate system. At a concentration of 2.1 × 10⁻⁴ M it decreased two-fold the conversion of liver lipids into thiobarbituric acid reactive substances (129). In another study 2.5 × 10⁻⁴ M ambroxol protected linoleic acid from peroxidative damage induced by the H₂O₂–Fe²⁺–EDTA system in vitro (126). At very high concentrations (millimolar range), which, however, are irrelevant for therapeutic use in humans, ambroxol completely inhibited -OH-induced peroxidation of linoleic acid as expressed by malondialdehyde (MDA) generation. The authors speculate that two mechanisms are responsible for these effects, first direct -OH scavenging effect of ambroxol and second the ability of the two nitrogen atoms of the drug molecule to chelate iron (130).

**AMBROXOL AS LIPID ANTI-OXIDANT IN ANIMAL STUDIES**

Ambroxol was demonstrated to reveal protective efficacy for lung and heart lipids that was comparable with and even higher than those of NAC and methionine (131–133). Ambroxol injected once per day (70 mg kg⁻¹) for three consecutive days decreased the LPS (17 mg kg⁻¹) induced lipid peroxidation in murine organs (133). The lung and heart levels of conjugated dienes (CDs) in animals pre-treated with ambroxol were 3.3 and 1.7 times lower than those observed in the control group which received buffer and subsequently LPS (132). NAC did not inhibit the LPS-induced (IL-1- and TNF-dependent) hyperthermic reaction in mice, while ambroxol inhibited the loss of body temperature from 41 ± 1.3 to 2.2 ± 0°C (132,133). These protective effects of ambroxol may result from its ability to scavenge -OH and the inhibition of IL-1 and TNF release from LPS-activated monocytes (126,134).

In another study mice were injected i.p. with equimolar doses (1.7 × 10⁻⁴ M kg⁻¹) of ambroxol, NAC, methionine or solvent once per day for three consecutive days. Lipid peroxidation was induced in organ homogenates by means of heat (50°C) or 10⁻⁵ M H₂O₂ (131). Ambroxol inhibited the thermal generation of CDs (one of the first lipid peroxidation products) in lung homogenates. This inhibition (59.9 ± 33.1%) was similar to that with NAC (61.1 ± 26.1%) but was 1.4-fold higher than that observed...
### Table 3. Effects of ambroxol *in vitro* and *ex vivo*

<table>
<thead>
<tr>
<th>Action</th>
<th>Concentration or dose</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Direct anti-oxidant activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- <strong>OH scavenger</strong></td>
<td>$10^{-3}$-$10^{-1}$ M</td>
<td><em>In vitro</em></td>
</tr>
<tr>
<td>- HOCl scavenger</td>
<td>$2.5$-$7.5 \times 10^{-3}$ M</td>
<td><em>In vitro</em></td>
</tr>
<tr>
<td>(2) Lipid anti-oxidant: inhibition of lipid peroxidation induced by</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- <strong>OH</strong></td>
<td>$2.5 \times 10^{-4}$-$5 \times 10^{-3}$ M</td>
<td><em>In vitro</em></td>
</tr>
<tr>
<td>- H$_2$O$_2$</td>
<td>i.p. $3 \times 70$ mg kg$^{-1}$</td>
<td><em>Ex vivo</em></td>
</tr>
<tr>
<td>- temperature, 50°C</td>
<td>i.p. $3 \times 70$ mg kg$^{-1}$</td>
<td><em>Ex vivo</em></td>
</tr>
<tr>
<td>- endotoxin (LPS)</td>
<td>i.p. $3 \times 70$ mg kg$^{-1}$</td>
<td><em>In vitro</em></td>
</tr>
<tr>
<td>- doxorubicin</td>
<td>i.v. $1 \times 70$ mg kg$^{-1}$</td>
<td><em>In vivo</em></td>
</tr>
<tr>
<td>(3) Inhibition of human phagocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- chemotaxis</td>
<td>$10^{-2}$ M</td>
<td><em>In vitro</em></td>
</tr>
<tr>
<td>- spontaneous migration</td>
<td>$9.6$-$3.8 \times 10^{-3}$ M</td>
<td><em>In vitro</em></td>
</tr>
<tr>
<td>- chemiluminescence</td>
<td>$9.6 \times 10^{-4}$ M</td>
<td><em>In vitro</em></td>
</tr>
<tr>
<td>- IL-1 and TNF release</td>
<td>$2.4 \times 10^{-5}$-$2.4 \times 10^{-4}$ M</td>
<td><em>In vitro</em></td>
</tr>
<tr>
<td>- modulation of intracellular Ca$^+$</td>
<td>$10^{-4}$ M</td>
<td><em>In vitro</em></td>
</tr>
</tbody>
</table>

The results obtained in the millimolar range are interesting from a scientific point of view but irrelevant in humans; because of low bioavailability it is not feasible to achieve concentrations of this order of magnitude by therapeutic administration.

**ANTI-INFLAMMATORY PROPERTIES OF AMBROXOL**

The inhibition of phospholipases A present in lung and alveolar lysosomes is an important mechanism by which ambroxol enhances the content of surfactant in the bronchoalveolar lining fluid (61). Ambroxol decreased the total activity of phospholipases A in rabbit alveolar macrophages, peritoneal cells and PMN cells (138,139). Furthermore, ambroxol at physiological concentrations (micromolar range) inhibited the spontaneous migration and chemotactic response of human PMN cells to purulent sputum, zymosan-activated serum and FMLP (60,140). Human monocytes cultured in medium containing ambroxol released less IL-1 and TNF after stimulation with LPS (134). The significant inhibition of biosynthesis of these two pro-inflammatory-pro-oxidant cytokines was observed just at an ambroxol concentration of $10 \mu$M. Ambroxol (2.4-$9.6 \times 10^{-5}$ M) attenuated the spontaneous and zymosan-induced lucigenin-dependent chemiluminescence of human PMN cells (6). This inhibition of phagocyte chemiluminescence may be a result of phospholipase inhibition with subsequent suppression of the respiratory burst and a direct scavenging effect of ROS. However, pre-incubation of human PMN cells and monocytes with ambroxol had no influence on phagocytosis and killing of *Candida albicans* (141). In dogs exposed to ozone aerosolized ambroxol inhibited the development of airway hyperresponsiveness to acetylcholine but did not decrease the PMN cell influx into the lower airways (147).

Table 3 summarizes the main effects of ambroxol indicating anti-oxidant and anti-inflammatory properties. Most of these effects came from *in vitro* or animal experiments using concentrations which are feasible to achieve (micromolar range) in human lung tissue (143).

**ANTI-OXIDANT FUNCTION OF AMBROXOL IN HUMANS**

Despite substantial evidence of the apparent anti-oxidative effect of ambroxol *in vitro* or in animals, only few studies in humans have been published. One describes that additional administration of ambroxol in cancer patients prevented deterioration of the lung function related to anti-cancer treatment with bleomycin and nitrosourea (144). Red blood cells of pre-term infants of mothers pre-treated i.v. with ambroxol revealed a decreased content of lipid for methionine. In addition, H$_2$O$_2$-induced generation of CDs in lungs from ambroxol-treated mice was 3-1-fold lower than that found in lung homogenates from the control group. In contrast, NAC and methionine failed to protect the lung lipids from H$_2$O$_2$-provoked peroxidative damage (131). This finding indicates that injection of ambroxol increases the lung anti-oxidant defence against heat and H$_2$O$_2$.

When applied first, a single intravenous injection of ambroxol (70 mg kg$^{-1}$) completely protected heart lipids from peroxidative damage induced by doxorubicin in mice (135). The heart content of CD and MDA in the animals that received ambroxol and doxorubicin was about three-fold lower than that found in the doxorubicin-treated mice. Ambroxol revealed also a protective action against other anti-cancer drugs and xenobiotics (*e.g.* bleomycin, paraquat) that are known to induce oxidative stress in tissues (136,137).
peroxidation products and enhanced anti-oxidant defence (145). While NAC cannot be detected in BAL fluid after i.v. or oral administration, ambroxol has a very high affinity for lung tissue, resulting in approximately 20 times higher levels in the lung than in serum. Thus, micromolar concentrations can be achieved in lung tissue after the administration of 1 g ambroxol (143). In a large double-blind multicentre trial 252 patients were enrolled receiving 1 g day⁻¹ of ambroxol i.v. for six consecutive days before upper abdominal surgery. In the lung tissue, resulting in approximately 20 times higher levels in the lung than in serum. Thus, micromolar concentrations can be achieved in lung tissue after the administration of 1 g ambroxol (143). In a large double-blind multicentre trial 252 patients were enrolled receiving 1 g day⁻¹ of ambroxol i.v. for six consecutive days before upper abdominal surgery. In this trial Feigz et al. demonstrated that there was a significant reduction in atelectasis between the ambroxol and the placebo groups (p＜0.05; ambroxol 10.6%, placebo 23.9%) (146). Furthermore, high doses of ambroxol (1 g day⁻¹) may prevent postoperative ARDS and broncho-pulmonary complications after chest surgery (147).


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