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Effect of oxidant air pollutants on the respiratory system: insights from experimental animal research

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Effect of oxidant air pollutants on the respiratory system: insights from experimental animal research. P. Chitano, J.J. Hosselet, C.E. Mapp, L.M. Fabbri. ©ERS Journals Ltd. 1995

ABSTRACT: In the present paper, we have reviewed experimental animal studies on the effects of the two most important oxidant airborne pollutants, nitrogen dioxide and ozone, on the respiratory system.

The toxic effects depend on concentration and length of exposure, and are generally similar for both oxidants, with ozone operative at lower concentrations. High doses of both oxidants cause death due to lung oedema Exposure to sublethal levels causes functional alterations such as airflow limitation and airway hyperresponsiveness to bronchoconstrictor stimuli. These effects, which are generally reversible, are associated with epithelial injury, oedema and airway and parenchymal infiltration by inflammatory cells. Loss of cilia of airway epithelium and necrosis of type I alveolar epithelial cells are the most prominent consequences at the epithelial level. Inmation is characterized by early neutrophilic infiltration, followed by an increased number of mononuclear cells, predominantly alveolar macrophages.

After long-term exposure, whilst nitrogen dioxide causes predominantly emphysema, ozone produces mainly pulmonary fibrosis. Biochemical effects include lipid peroxidation, increased antioxidant metabolism, and alteration of enzyme activity. Nitrogen dioxide and ozone may also alter the immunological response and reduce the defence against infections, increasing the susceptibility of exposed animals to infections.

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CONTENTS

Introduction

Environmental pollution has become, in the last few decades, one of the most prominent concerns for human health. As demonstrated by serious incidents which occurred in the past, air pollution can represent a major health hazard and cause an increase in mortality of the exposed population, mainly due to bronchitis, pneumonia, and exacerbation of pre-existing heart or lung diseases [l–3].

The actual impact of pollutants on health is related to the extent of individual exposure, which depends on factors such as the location and character of the sources, the spread and properties of the pollutants, smoking habits of the individuals, and other activities that increase contact with hazardous substances [4, 5]. All these factors need to be taken into account, especially in epidemicological studies [6, 7].

Experimental studies on humans allow carefully controlled exposures. Since subjects obviously cannot be exposed to unsafe levels of pollutants in these studies, low and short-term exposures are utilized, providing knowledge about the first stages of disease development only. Studies on animals are the most appropriate for the examination of the mechanisms underlying functional, structural and biochemical alterations caused by acute and/or chronic exposures to the widest range of pollutant concentrations. The major drawback of animal studies is species variability, often making extrapolation of animal results to man difficult. Both research on animals and data from human experimental and epidemiological studies are, therefore, complementary in our understanding of lung injury induced by air pollutants, and taken together, may contribute to improve prevention and management [7, 8].

In the present review, we analyse experimental studies on animals, focusing on nitrogen dioxide (NO_2) and ozone (O_3), since these two oxidants are the most common airborne pollutants, and have been shown to have several severe adverse respiratory effects.

Sources and distribution of oxidant air pollutants

The release of potentially toxic chemicals into the air can result both from natural and anthropogenic events. The level they reach in a given environment is dependent on the number and location of sources, on the quantity of pollutants released, and on their subsequent fate in the air. Because of the number of different sources, NO_2 and O_3 reach highest concentrations in urban areas [9, 10].

Nitrogen dioxide is formed by the oxidation of nitrogen oxide, that is generated from oxygen and nitrogen by processes involving high temperatures. It is a product of most operations requiring combustion, and is present in motor vehicle emissions [9]. NO_2 is also a major component of indoor air pollution, as it is generated by cigarette smoking and during the combustion of natural gas and kerosene, frequently used as energy sources for cooking and heating [5, 11].

Ozone forms naturally at high altitudes (above 20 km) as a result of the action of ultraviolet radiation on molecular oxygen, which dissociates to oxygen radicals, that in turn react with other oxygen molecules to form O_3. The energy of ultraviolet radiation which penetrates at lower altitudes is not sufficient to dissociate molecular oxygen, but is enough to dissociate NO_2, when present, to form nitrogen oxide and oxygen radicals. In these conditions, the reaction between oxygen radicals and molecular oxygen to form O_3 may also occur at ground level. Moreover, the release of unburnt hydrocarbons by combustion processes may further oxidize nitric oxide, increasing the level of NO_2 and, consequently, the O_3 concentration in the air [10, 12].

Once toxic air pollutants are produced or released, their environmental concentration varies according to transport and diffusion from the source and the extent and velocity of the transformations they undergo [5, 9, 10, 12]. NO_2 environmental concentration generally remains under 5 parts per billion (ppb) in rural zones, whereas it ranges between 10–75 ppb, with peak values of up to 500 ppb in urban areas, both outdoors and indoors [5, 9]. It reaches extremely high values (up to 500 parts per million (ppm)) in those zones of workplaces where welding arcs or blow torches are employed [13]. The level reached by O_3 is normally under 50 ppb in unpolluted air, whilst concentrations of up to 700 ppb have been found in the air of polluted cities [9, 12].

Present recommendations state that the levels of environmental NO_2 should not exceed 210 and 80 ppb for more than 1 and 24 h, respectively, [14]. Occupational threshold limit values (TLVs) for NO_2 vary in different countries between l–5 ppm time-weighted average (TWA) concentration, and between 3–10 ppm short-term exposure limit (STEL) [15]. O_3 in the general environment should not reach concentrations higher than 100 and 60 ppb for more than 1 and 8 h, respectively, [14], whilst occupational TLVs for O_3 are 100 ppb TWA and 300 ppb STEL [15].

Animal exposure and oxidant uptake

The levels and kinds of hazard which develop at a given target site depend on the amount of pollutant and on the contact time at that site. Therefore, to estimate the potential effects of air pollutants, those factors which contribute to exposure and uptake need to be carefully considered. Control of pollutant concentration and length of exposure are easily achieved in experimental studies. Once these are fixed, several other factors may affect the dose of a given pollutant that reaches a specific site of the respiratory system. Spontaneous breathing through the nose or the mouth and mechanical ventilation are two experimental conditions in which the surface of respiratory system exposed to the inspired air is greatly different: intubation permits a higher concentration of pollutant to reach central and peripheral airways. Exercise is a further condition which, mainly altering the ventilation, changes the dose of pollutant delivered to a given site. The anatomy and mechanics of the lung vary greatly from species to species and, thus, the pollutant uptake may also vary greatly in different species [7, 8, 16, 17].

The distribution of a gaseous component of inhaled air within the respiratory system also depends on the physicochemical properties of that component, particularly on its solubility and reactivity [8, 16–18]. A soluble gas is quickly removed from inspired air into the first tissue it meets, so that its uptake occurs predominantly in the upper airways. By contrast, the uptake of insoluble gases is due mainly to their reactivity, which is related to substrate availability and exposed surface, and is, therefore, more uniformly distributed along the respiratory system.

Since O_3 and NO_2 are gases with low solubility, they are poorly absorbed by the airway mucosa [16–18]. Nevertheless they are very reactive molecules and their uptake in the respiratory system is extremely high [19–22]. Experimental measurements performed in dogs have shown that the uptake of O_3 in the upper airways may be as high as 70% of the inspired quantity at 0.3 ppm concentration and 4.5 $L \cdot min^{-1}$ flow, but it decreases to below 30% at a concentration and flow of 0.8 ppm and 40 $L \cdot min^{-1}$, respectively. O_3 uptake may be as high as 80% in the lower airways, where it is independent of inlet flow and concentration. Combining data for upper and lower airways, an extrathoracic removal of O_2 of about 40% and a total uptake exceeding 90% has been estimated [19]. Slightly lower values of total absorption (almost 70% of inlet concentration) have been reported for NO_2 in ventilated monkeys and in isolated rat lung [21, 22].

Mathematical dosimetry models have been developed to explain, simulate, and predict regional uptake of inhaled gases [12, 17, 18]. These theoretical models take into account lung anatomy and mechanics, and the physicochemical properties of the respiratory system as well as of gas pollutants. Comparison between experimental data and predicted values have been used for the validation of the effectiveness of such models. The use of these models suggests that the uptake both of NO_2 and O_3 occurs to a similar extent between the trachea and the respiratory zone, and peaks at the terminal bronchioles.

Besides the site of absorption, several data reveal the role of chemical reactivity in the uptake and toxicity of these air pollutants.

UsingO_2 and oxygen, both labelled with ^{18}O, it has been shown that O_2 determines a 15,000 times greater uptake of ^{18}O, than molecular oxygen, and that tissues in closer contact with the inhaled gas (*e.g.* epithelium) carry a higher dose of ^{18}O [20, 23]. O_3 absorption increases with temperature and is not dependent on vascular perfusion, suggesting that its uptake into the lung tissue is rate-limited by chemical reaction rather than by physical solubility [24]. In fact, whilst chemical reactivity increases with temperature, physical solubility decreases with temperature. In addition, solubility increases if the removal of solute is faster, *e.g.* when vascular perfusion of the lung is higher. Therefore, it is likely that O_3 reacts entirely in the epithelium lining fluid, generating diffusing products such as aldehydes, hydrogen peroxide and free radicals, which in turn account for the toxic effects that follow O_3 exposure [18, 23, 25, 26].

Like O_3, NO_2 uptake increases with temperature and is not dependent on vascular perfusion [21]. NO_2 uptake occurs predominantly in the epithelium lining fluid [21, 23, 27–29], where NO_2 reacts and produces nitrite, nitric and nitrous acids, which can themselves cause oxidative damage [22, 28, 29]. The formation of these products would explain some distinctive effects of NO_2, such as those on mucociliary clearance and macrophage phagocytic activity in rabbits, which are intermediate between the effects exerted by a pure oxidant (O_3) and those exerted by a pure acid (H_3SO_4)[29].

Toxicity

Several acute and chronic effects determined by oxidant gases have been reported, which affect function, structure, and biochemistry of the respiratory system, involving inflammatory and immunological responses.

Very high doses of NO_2 cause the death of exposed animals in a few minutes [13, 30, 31]. In rats, the concentration which kills 50% of exposed animals after a given time of exposure (LC_{50}) is 1,445 ppm NO_2 for 2 min of exposure, 416–833 ppm for 5 min, and 115–168 ppm for 1 h of exposure [30, 31]. LC_{50} for 1 h exposure is 21 ppm O_3 in mice and rats, and 52 ppm O_3 in guinea pigs [32].

Irrespective of the oxidant concentration and of the duration of exposure which give rise to the fatal event, for both oxidants the cause of death is attributed to acute lung oedema.

Effects of lung function and airway responsiveness

Exposure to low levels of O_3 and NO_2 may alter respiratory function in several animal species. These changes of lung function consist of increased respiratory frequency, decreased tidal volume, air trapping, airflow limitation, and increased airway responsiveness to contractile agents.

Lung function. Functional alterations have been reported after exposure to concentrations as low as 0.24–0.34 ppm O_3. Acute exposure increases respiratory frequency and decreases tidal volume [12, 33–35]. These effects are inhibited by bilateral cooling of the cervical vagi. They have, therefore, been attributed to an altered sensitivity of the vagal bronchopulmonary receptors, most likely of C-fibre endings and rapidly adapting irritant receptors [36], with increased reflex stimulation of the diaphragm through the phrenic nerve [37].

In addition, acute exposure to O_3 may cause air trapping, as shown by increased functional residual capacity and increased closing volume. Air trapping is probably due to premature closure of the small peripheral airways. Ozone may also cause acute airflow limitation, as shown by increased airway resistance and reduced dynamic compliance. These acute obstructive effects quickly reverse after cessation of exposure [12, 38, 39]. Airflow limitation is also observed after chronic exposures, mainly caused by fibrotic lesions, and in these cases it persists after cessation of exposure [12].

NO_2 exposure at concentrations of 0.8–5 ppm has similar effects on mechanical lung parameters [13, 33, 40, 41]. The severity of these effects is influenced by the mode of exposure. Indeed, when compared to continuous exposure, a greater reduction of end-expiratory volume, vital capacity, and respiratory system compliance has been shown in mice chronically exposed to 1 h spikes of 0.8 ppm NO_2 superimposed on a continuous baseline exposure to 0.2 ppm [42].

Airway responsiveness. A reversible increase in airway responsiveness to several contractile agents is observed in animals after acute exposure to a concentration of O_3

ranging 0.5–3 ppm [35, 38, 43–52]. Increased airway responsiveness to histamine has also been reported following exposure to 4 ppm or higher concentrations of NO_2 in guinea-pigs [53–54]. No study has been performed on the mechanisms of NO_2–induced airway hyperresponsiveness, whereas several mechanisms have been shown to be involved in the O_3-induced airway hyperresponsiveness.

Pretreatment with inhibitors of oxygen radical generation prevents the development of airway hyperresponsiveness to acetylcholine in dogs exposed to O_3 [55]. This finding suggests that the generation of free radicals is involved.

The increased airway responsiveness to acetylcholine in dogs can also be inhibited by the administration of different anti-inflammatory agents: *e.g.* BW755C [56], an inhibitor of arachidonic acid metabolism; indomethain [57], a cyclooxygenase inhibitor; OKY-046 [58], a thromboxane synthase inhibitor; and ambroxol [59], a surfactant-stimulating agent, which has also been shown to inhibit phospholipase A. In guinea-pigs, O_3-induced airway hyperresponsiveness to methacholine is inhibited by OKY-046 and ONO-1078 (a receptor antagonist of leukotriene C_4 and D_4 (LTC_4 and LTD_4)) [60]. These data suggest that oxygenation products of arachidonic acid play a role in O_3-induced airway hyperresponsiveness to cholinergic stimuli.

Moreover, it has been reported that exposure of guinea-pigs to 0.1 ppm O_3 causes an inhibition of lung cholinesterase activity, which may contribute to the development of cholinergic hyperresponsiveness [61].

A recent study on the guinea-pig has shown a further mechanism for the increased cholinergic responsiveness: a loss of function of M_2 muscarinic neural receptors after 4 h of exposure to 2 ppm of O_3 [62]. These receptors normally act as feedback inhibitors on the release of acetylcholine from nerve endings, so that a potentiation of vagally-mediated bronchoconstriction occurs when they are inhibited.

The mechanisms responsible for cholinergic hyperresponsiveness may also apply to the O_3-induced increased airway responsiveness to other stimuli. Indeed, pretreatment of dogs with atropine-sulphate aerosol or cooling blockade of conduction of the vagus nerves have been shown to reduce the O_3-induced hyperresponsiveness to histamine, suggesting that this hyperresponsiveness may be mediated, at least in part, *via* cholinergic pathways [45]. However, in guinea-pigs exposed to O_3. the development of lower airway hyperresponsiveness to intravenous administration of histamine is not suppressed by bilateral cervical vagotomy or by inhibitors of arachidonic acid metabolism [52], and thus noncholinergic components may also mediate the hyperresponsiveness to histamine.

The response to platinum, ovalbumin and Ascaris is enhanced by O_3 in platinum-sensitized monkeys, ovalbumin-sensitized guinea-pigs and Ascaris-sensitized dogs, respectively, [48–51]. Like the hyperresponsiveness to intravenous histamine, the increase in airway responsiveness to Ascaris antigen induced by ozone in *Ascaris suum* allergic dogs is not inhibited by the thromboxanesynthase inhibitor OKY-046, suggesting that thromboxane may not be involved [48]. This same study showed an increased plasma histamine concentration after antigen challenge in animals exposed to O_3 compared to control dogs. Since the release of histamine from mast cells is an important component of the smooth muscle response to allergen challenge, it is not surprising that the O_3-induced hyperresponsiveness to a specific antigen shows similarity to the O_3-induced hyperresponsiveness to histamine, *i.e.* a lack of involvement of arachidonic acid metabolites.

A further different mechanism might be responsible for the O_3-induced airway hyperresponsiveness to substance P: a reduced activity of the neutral endopeptidase, the enzyme which cleaves substance P in addition to other tachykinins [47, 63]. Indeed, neutral endopeptidase activity in tracheal tissue homogenate is significantly reduced in the guinea-pig by 2 h of exposure to 3 ppm O_2 [53].

Histopathology

A histological approach reveals the differences, resulting from the length and amount of exposure, in the patterns of either NO_2- or O_3-induced lung injury. An exposure of between 1 h and 1 week, which is considered a short-term (acute) exposure, induces cell injury, lung oedema and inflammation, which may be reversed after cessation of the exposure. Some of these effects decline with time even upon continuation of the exposure. A long-term (chronic) exposure (from one month to years) can also induce irreversible lesions, such as emphysema and pulmonary fibrosis.

Structural damage. Structural damage is induced both by acute and chronic exposure to NO_2 or O_3, mainly affecting cells at the junctions of the terminal airways and the proximal alveolar regions. It includes loss of cilia and secretory granules, necrosis and sloughing of epithelium [12, 13, 41, 64–80].

The threshold concentrations of oxidants which cause structural alterations are related to duration of the exposure. In fact, the same structural changes may be caused by concentrations of NO_2 and O_3 of, respectively, 10 and 0.5 ppm after acute exposures, and 1 ppm of NO_2 and 0.06 ppm (with spikes of 0.25 ppm) of O_3 after chronic exposures.

Loss of cilia in the terminal bronchioles is considered an early indicator of acute oxidant-induced injury, even though it is also consistently found after long-term exposure [41, 64–72]. Necrosis of airway epithelial ciliated cells in terminal airways and of type I alveolar epithelial cells in centriacinar regions constitutes the main cellular alteration [65, 69–76]. Other morphological changes frequently reported are damage to tight junctions and to Clara cells and goblet cells, with loss of secretory granules [66, 71, 72, 79], as well as thickening of the epithelium and of the alveolar wall with hypertrophy and hyperplasia of epithelial cells, particularly Clara cells and alveolar type II cells [65–80]. After either subchronic or chronic exposure, hypertrophy of smooth muscle and endothelial cells, hyperplasia of goblet cells and accumulation of mucus are also found in respiratory bronchioles [65, 76–78].

These injuries may lead to accumulation of debris in the airway lumen, and, therefore, to bronchiolitis oblit-

erans and obstructive inflammatory lesion in bronchioles, with narrowing of the small airways [65, 68, 71, 76, 78].

Lung oedema. Exposure to high concentrations of NO, or O_3 cause acute fatal lung oedema. Lung oedema may also occur after exposure to sublethal levels. A macroscopic consequence of oedema formation after exposure to these two oxidants consists of increased lung volume and wet weight [32, 76, 77, 81]. A sensitive index of lung oedema, which constitutes a common finding after exposure both to NO_2 and O_3, is the presence of serum proteins in the bronchoalveolar lavage fluid [82–85]. Alteration of epithelial and vascular permeability leading to oedema is particularly marked at small airway and alveolar levels [75, 85–88]. Morphological observations show mainly focal interstitial and intra-alveolar oedema [75,87], with the former more pronounced around arteries than airways or veins [89]. A strong correlation has been found between the degree of NO_2 or O_3 exposure and the amount of oedema observed both in the alveolar spaces [88, 89] and in peribronchiolar and perivascular tissue [81, 84, 89]. Oedema is more evident at an early stage of exposure [75, 90], but has also been reported after long-term exposure [78]; it reverses after cessation of exposure [84, 87, 88].

Inflammatory response. Inflammation is a common feature of the lung response to a variety of environmental noxious agents, and has been noted in many species after exposure to O_3 or NO_2 [49, 65, 81, 82, 91–95].

The activation of resident macrophages and the chemotaxis of inflammatory cells into the lung is determined by preformed and/or newly formed mediators released from different cells in response to injury. Activated alveolar macrophages and neutrophils generate oxidizing products, which interact with both proteinases and proteinase inhibitors, producing additional damage to connective tissue, endothelial and epithelial cells. This results in amplification of oedema, injury, airway responsiveness and inflammation [96–98]. Therefore the direct toxic effects of oxidant gases may be potentiated by the release of mediators and the production of oxidant species from inflammatory cells.

Inflammation caused by exposure to oxidants occurs both in the airways and in the pulmonary tissue and may be investigated with different techniques, *e.g.* bronchoalveolar lavage and histopathological examination of necropsy or biopsy specimens.

Several reports have shown that recruitment of different cell populations into the lung and the airways occurs at different time-points after exposure. Terminal bronchioles and alveolar regions have been described as the predominant, or at least the initial, site of cellular infiltration [66, 71, 80–82, 86, 95].

In rabbits, it has been shown that the number of cells obtained by bronchoalveolar lavage increases both after acute and subacute exposure to O_3 (to 1.2 and 0.1 ppm, respectively), *i.e.* neutrophils increase predominantly at 24 h after exposure, and alveolar macrophages at 7 days after exposure [92]. The intravenous infusion of labelled neutrophils at different time-points after exposure to 0.96 ppm O_3 has recently been used on Rhesus monkeys to demonstrate the time-course of migration into the lung [95]. The study showed that neutrophil migration, as revealed by bronchoalveolar lavage, is maximum 12 h after the exposure and returns to baseline within the next 12 h; after migration, neutrophils persist in the airways for up to 72 h, at which point they are reduced to a quarter of the maximum amount. Neutrophils and macrophages have been observed on the luminal surface and in the walls of terminal bronchioles, after 8 and 24 h of exposure, respectively [82, 95]. In the same studies total protein content in bronchoalveolar lavage was maximum at 24 h, and lymphocytes migrated maximally about 72 h after the exposure.

Lymphocyte infiltration of the lung tissue as well as T-lymphocyte proliferation in lymph nodes and in bronchus-associated lymphoid tissue have been found in histological specimens after O_3 exposure [99, 100]. These reports suggest that T-cells may play a role in the host response to oxidant gases.

Increased protein concentration, neutrophils, lymphocytes and macrophages in bronchoalveolar lavage fluid and in lung tissue have also been reported after exposure to NO_2 [66, 81, 86, 91, 101, 102]. In short-term exposure to NO_2, patchy interstitial accumulation of neutrophils can be observed in the bronchial walls [86, 91]. The initial response to 30 ppm of NO_2 consists of a marked increase in neutrophils, with a peak after 2 days of exposure, whereas macrophage and lymphocyte infiltration is maximum after one week of exposure or more [102]. An influx of alveolar macrophages and lymphocytes has been reported following both acute and chronic exposure to NO_2 [66, 70], the degree of infiltration being dependent on gas concentration and duration of exposure [81, 86, 101].

Relationship between airway permeability and airway inflammation. Tracheal permeability develops immediately after exposure to oxidant gases [83–88, 103–106] and reaches a peak at 8 h postexposure, whilst the number of polymorphonuclear leucocytes usually peaks at 12 h [103]. Rats rendered leukopenic with cyclophosphamide have a significant reduction in circulating and pulmonary leucocytes and in airway permeability induced by O_3, and pretreatment with either the LTD_4 antagonist FPL55712 or indomethacin also inhibits the O_3-induced increase in permeability [104]. By contrast, when neutrophil depletion is obtained with a rabbit anti-rat neutrophil serum, neutrophil-depleted rats exposed to 1 ppm O_3 have a significant increase of protein in bronchoalveolar lavage [85]. Moreover, cyclophosphamide, indomethacin and colchicine (an inhibitor of chemotaxis) prevent cell migration into the lung, but do not reduce airway permeability induced by O_3 in mice [105]. These data suggest that cellular influx and increased lung permeability may be independent parts of the lung inflammatory response to O_3.

Relationship between airway inflammation and airway responsiveness. After exposure to O_3, the development of airway inflammation has a time-course similar to that of the development of airway hyperresponsiveness [93, 94]. Neutrophil depletion by hydroxyurea inhibits airway hyperresponsiveness in dogs [107]. Inhibition of O_3-induced airway hyperresponsiveness by different

agents has also been achieved, without a reduction of the number of inflammatory cells in the airway mucosa [55, 57–59]. These results suggest that the inhibition of O_3-induced airway hyperresponsiveness may be mediated by the inhibition of the release of mediators by inflammatory cells. The prevention of neutrophil migration by monoclonal antibodies against neutrophil adhesion molecules does not affect the O_3-induced increase in airway responsiveness [108]. Moreover, exposure of guinea-pigs to 2.9 ppm O_3 for 30 min increases airway responsiveness to methacholine at 1 h, without signs of inflammation in bronchoalveolar lavage [44]. The same authors found bronchoalveolar neutrophilia at 3 and 6 h after exposure. From these reports it seems that noninflammatory cells are the most likely candidates either for the production of newly formed mediators or the release of preformed ones which determine the increase in airway responsiveness; inflammatory cells may still play a potentiating role in the development of airway hyperresponsiveness after exposure to O_3.

Emphysema and pulmonary fibrosis. With long-term exposure to oxidants, emphysematous and fibrotic lesions have been reported. However, these chronic effects differ with oxidant agents. Indeed, chronic exposure to NO_2 produces both fibrosis and emphysema of the lung, whereas emphysema-like injuries have not been described after O_3 exposure.

In long-term exposure to NO_2, the respiratory bronchiole segment increases in size and alveolar ducts and alveoli show focal enlargement with destruction of alveolar septa [68, 76, 77, 86], as in human centriacinar emphysema. Therefore, NO,-induced emphysema has been used as an animal model to study the disease process of emphysema [109, 110]. An increase in lung volume and a decrease of the number of interalveolar septa, which are considered as markers of emphysema, are observed within 3–6 weeks of exposure to 30 ppm NO_2 [86, 111]. Some of the above-reported studies have also shown that fibrotic lesions may be present in the lung, associated with the emphysematous ones, after long-term exposure to NO_2 [77, 86].

A regional structural remodelling of the central acinus has been observed after long-term exposure to O_3 [78], which is characterized by fibroblast proliferation, thickening of the alveolar capillary membrane, increased synthesis and deposition of collagen in different regions of the lung, thicker walls and reduced internal diameter in terminal bronchioles, and fibrosis of interalveolar septa [32, 75, 78, 112, 113].

Repair. Once the exposure is over, most lesions caused by acute exposure to oxidant gases reverse. The reparative process may, in fact, start early during exposure. In alveoli, damaged epithelial cells are shed and are replaced by other cells, mainly type II cells [67, 69, 75, 114]. In bronchioles, Clara cells divide and differentiate into ciliated cells [67, 69, 70, 75, 115]. Cell proliferation has been shown to reach a maximum after 24–48 h exposure to 17 ppm NO_2 and then to decline [67]. Recovery occurs more promptly in major bronchi than in terminal bronchioles, so the particular sensitivity of terminal bronchioles to oxidant gases may be explained by a lower reparative ability at this site of the airways [71]. Most of the early alterations which occur as a result of the exposure to 10–20 ppm NO_2 are likely to recover totally after the cessation of exposure [70, 114].

After repair of the injury, animals can become tolerant to further continuous exposure of a similar entity to the previous one [12, 114]. Furthermore, in continued exposure, a reduced level of inflammation has been observed after the first week [75, 86]. In this respect, the fact that NO_2-induced emphysema is not aggravated by prolonged exposure [86], has been suggested to be due to the decrease in the number of neutrophils and to the increase of antioxidant activity with time.

Biochemical alterations

The biochemical alterations which occur in the respiratory system following exposure to oxidants include changes in lung lipids, antioxidant metabolism, and enzyme activity. Membrane polyunsaturated fatty acids and thiol groups seem to be the main biochemical targets of NO_2 and O_3.

Lipid peroxidation and antioxidative protective system. Both after short- and long-term exposure to NO_2 or O_3 at concentrations varying from 0.04 to 10 ppm, fatty acid peroxidation, which is considered an integral part of cell damage, has been demonstrated by the use of several markers of this reaction. Ethane exhalation in the breath has been measured in living animals, whilst in tissue homogenate the determination of the amount of tissue which reacts with thiobarbituric acid and of the content of conjugated dienes have been employed [116–121].

It has been suggested that the induction of the antioxidative protective system may be a compensatory cell mechanism against lipid peroxidation [116]. Indeed, the stimulation of the glutathione peroxidase system and of other protective enzymes, such as superoxide dismutase, disulphide reductase and 6-phosphogluconate dehydrogenase, is a response to short- or medium-term exposure to oxidants [65, 84, 91, 101, 116, 122, 123]. By contrast, this antioxidative protective enzymatic system is inhibited after long-term exposure to low levels of NO_2 [117]. Therefore, one might speculate that the time-course of such enzymatic changes may explain both why epithelial cells become more tolerant to oxidant gases after short- and medium-term exposure *(i.e.* during the stimulatory phase of protective enzymes) and why there is the development of emphysema and/or fibrosis after chronic exposure to NO_2 *(i.e.* when the antioxidative protective enzymatic system is inhibited).

Membrane lipid peroxidation after exposure to NO_2 is associated in rat airways with variations of vitamin E and nonprotein sulphydryl contents [116, 122]. Vitamin E administration may partially protect the cell from peroxidation induced by O_3 and NO_2, whereas its depletion increases peroxidative effects, probably because of its modulatory effect on the activity of antioxidative enzymes [80, 120, 124].

Other enzymatic activity. Other enzymatic alterations

induced by oxidants involve the arachidonic acid cascade and the elastase-antielastase system.

Direct oxidant action and the products of peroxidative injury activate phospholipase A_2 [125], and liberate arachidonic acid metabolites, such as LTB_4 [126], prostaglandins E_2 and $F_{2\alpha}$, and thromboxane B_2 [127], which may play a role in the inflammatory response and in the induction of hyperresponsiveness.

The results of several studies suggest that an imbalance in elastase/antielastase is involved in the induction of the NO_2-induced emphysema [86, 102, 111, 128–130]. Elastase release contributes to lesion development through connective tissue destruction, especially when the elastase inhibitory activity (most likely due to $alpha_1$-proteinase inhibitors) is reduced. In rats chronically exposed to NO_2, GLASGOW *et al.* [86] observed that the marked neutrophil recruitment was concomitant with an increased neutrophil elastase burden in the lung, which may directly contribute to lesion development. In hamsters, KLEINERMAN *et al.* [102] demonstrated an increased elastolytic activity dependent on increased number of alveolar macrophages recovered in bronchoalveolar lavage fluid from animals exposed to 30 ppm of NO?. In both studies, the elastolytic secretion per cell seemed to be unaffected by exposure to NO,. Elastin content of the lung has been shown to decrease within 10 days during NO_2 exposure [128] and its increased degradation can be detected by higher urinary desmosine levels [129]. Moreover, the soluble fraction of lung collagen increases during NO_2 exposure, whereas the insoluble fraction, as well as total collagen, decreases [128, 130], suggesting that NO_2 enhances the catabolism of lung collagen [130].

The results of these studies suggest that NO_2-induced emphysema may be due to an impairment in elastase-antielastase. However, elastase-induced emphysema is worsened by the administration of beta-aminopropionitrile, an inhibitor of the lysyl oxidase, which in turn mediates the cross-linking reaction between collagen and elastin [109]. By contrast, aminopropionitrile neither increases the severity nor accelerates the timecourse of NO_2-induced emphysema [111], suggesting that NO_2 probably causes emphysema by inhibiting lysyl oxidase.

Exposure to O_3 has been shown to produce an increase in the collagen synthesis and deposition, without effects on elastase activity and elastin content [32, 131, 132]. Since an increase in collagen is associated with fibrotic lesions, these observations may explain why fibrosis rather than emphysema develops in response to this oxidant.

Impairment of lung defence

Exposure to oxidants has been shown to affect lung defence mechanisms, as demonstrated both by a greater susceptibility to infections and altered immunological response.

Defence against infections. A reduced efficiency of the defence against infections is a well-documented effect of exposure both to O_3 and NO_2 at concentrations as low as 0.08 and 0.5 ppm, respectively, [133, 134].

Exposure to NO_2 or O_3 determines a dose-dependent increased susceptibility to and mortality from infectious agents [133–138]. A quantitative bacteriological monitoring of mice lung tissue has been used, which consists of infecting the animal with bacteria labelled with radioactive phosphorus, and of subsequent simultaneous measurement of pulmonary radioactive phosphorus and of bacterial concentrations. With this technique, it has been shown that lungs experimentally infected with labelled *Staphylococcus aureus* exert a reduced antibacterial activity when exposed to NO_2 either before or after being infected [139]. The cellular target of NO_2 and O_3 exposure involved in pulmonary infections appears to be the alveolar macrophage [92, 138, 140, 141], which shows, both after single and combined exposures to O_3 and NO_2, changes in size and surface morphology, as well as decreased viability, substrate attachment ability, and decreased phagocytic and killing activity [92, 101, 138, 141, 142]. Moreover, the damage to ciliated epithelial cells described previously contributes to this NO_2- and O_3-induced impairment of the defence against infections.

Immunological response. It has also been reported that O_3 and NO_2 exposure can alter the pattern of the immunological response [136, 143–147]. An increased level of immunoglobulins in lung lavage fluid has been reported in mice after exposure to O_3 [143]. In guinea-pigs, the appearance of serum antibody to normal lung tissue relates to the amount and duration of exposure to NO_2, possibly as a consequence of an auto-antigenic effect of altered components of the lung tissue [144]. Furthermore, an altered antibody production has been observed in response to different antigenic stimuli [145–147]. These studies report either suppression or increase of the antibody responses. Contradictory results have also been obtained on cell-mediated immunological function, since both suppression and elevation of cellular responses have been observed [148, 149]. The actual effects of exposure to oxidants on the immune system remain to be understood; the observed discrepancies may depend on the mode of exposure, animal species or antigen employed.

O_3 exposure has also been shown to reduce the antigen sensitization threshold in guinea-pigs [51]; animals exposed to 5 ppm O_3 before sensitization need less ovalbumin to reach sensitization and show greater antibody production. Moreover, as reported above, when they are exposed after sensitization, the response to airway challenge occurs at lower antigen doses [49, 51]. Facilitation of sensitization to ovalbumin has also been reported after exposure to 40 ppm NO_2 [150].

Synergistic effects

Interactions among toxic components of air pollution may occur, giving additive or synergistic (more than additive) effects. Potential interactions between O_3 and NO_2 and between each of these two oxidants and other pollutants at environmentally relevant concentrations should, therefore, be evaluated when setting air quality standards. Indeed, several animal studies have shown that synergistic toxicity does exist for most of the different effects exerted by these two oxidants.

Simultaneous inhalation of 0.4 ppm O_3 and 7 ppm NO_2 for 6 h in rats causes a more than additive increase of epithelial cells recovered in bronchoalveolar lavage; permeability and inflammation (measured as protein and polymorphonuclear cells in the bronchoalveolar lavage fluid) are also synergistically augmented by simultaneous inhalation of slightly higher concentrations of oxidants (0.6–0.8 and 1l–14 ppm of O_3 and NO_2, respectively) [151].

Biochemical studies have demonstrated that both peroxidation and enzymatic activity (superoxide dismutase, glutathione reductase and peroxidase, 6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase) are potentiated when NO_2 and O_3 are administered simultaneously [118, 119, 152]. These biochemical responses are evoked even by exposures to 0.4 ppm or less of NO_2 and O_3, concentrations at which both oxidants are unable to exert any effect on their own. An increased production of prostaglandins E_2 and $F_{2\alpha 5}$ is also potentiated by exposure to a mixture of the two oxidants [127]. Moreover, some studies have shown that the effects exerted by NO_2 and O_3 on rate of collagen synthesis may be potentiated by simultaneous administration of acidic aerosols [112, 113].

Mortality caused by experimental infections with pathogens has also been studied following exposure to a mixture of NO_2 and O_3, and a synergistic effect has been observed, possibly because of the synergism exerted on the factors described above [136].

In vitro studies

The use of several techniques of investigation *in vitro* may contribute to a deeper understanding of the mechanisms underlying oxidant gas-induced injury. Effects on cells in culture and on whole isolated tissues have been widely analysed, focusing on the different aspects reviewed above, and have clarified some of the links between effects observed *in vivo*.

Cell culture and mediator release

To investigate the primary interaction occurring between cells and gases and the subsequent pathogenic events, *in vitro* systems in which isolated cells are exposed to oxidant gases have been developed [153–155]. The cell response to gases may be amplified by variations of cell culture conditions [156, 157], so that comparison and extrapolation of results from these experiments to the *in vivo* exposures may be complicated. Nonetheless, similar results have been obtained by a comparative study on phagocytosis of alveolar macrophages obtained from rats exposed to 4–25 ppm NO_2 *in vivo* and macrophages exposed *in vitro* to comparable gas concentrations [158].

Reduced viability after *in vitro* exposure to O_3 has been observed on cultured cells [159–161]. Exposure to O_3 induces cytoplasmic vacuolization, cell necrosis and lipid peroxidation in monolayer cultures of tracheal epithelium [161]. Lipid peroxidation and an alteration of membrane fluidity and properties after exposure to O_3 and NO_2 have been reported in epithelial and endothelial cells [161–163], fibroblasts [164], and macrophages [159, 165]. Impaired membrane function seems, therefore, an important aspect of oxidant-induced injury, as it may be the primary event of the development of pulmonary oedema.

Using alveolar macrophages cultured in aerobiosis, *i.e.* layered on porous membranes saturated by capillarity with culture medium and kept in direct contact with the atmosphere, it has been observed that cytotoxicity of oxidant gases, evaluated by morphological changes and by adenosine triphosphate cell content, is modulated by the antioxidant enzyme equipment and glutathione cell content [166]. Increase of phagocytic activity and of cytotoxicity toward mammary adenocarcinoma cells have been reported both in alveolar macrophages exposed to 10 ppm NO_2 *in vitro* and in cells obtained from animals exposed *in vivo* to 40 ppm [167]. By contrast, after longer *in vitro* or *in vivo* exposures to oxidant gases, alveolar macrophage superoxide anion production, mobility, phagocytosis, and bacterial killing decreases [92, 154, 158, 159, 168].

The involvement of arachidonic acid metabolites in oxidant-induced injury is supported by *in vitro* studies, which show increased phosphatidylserine content and specific activation of phospholipase A in plasma membranes [1691, formation of cyclooxygenase and lipoxygenase product in epithelial cells [170, 171], and a concentration-dependent increase in release of prostaglandin E_2 in alveolar cells [168, 172, 173]. Since exogenous prostaglandin E_2 has been demonstrated to inhibit macrophage phagocytosis, it conceivably plays a role in the reduction of macrophage phagocytic activity induced by O_3 [172]. Moreover, after NO_2 exposure in standard cell cultures, alveolar macrophages have been shown to generate a neutrophil chemotactic factor identified as the LTB_4 [126].

These data suggest that cell damage is a direct oxidant effect and that the oedema formation and the inflammatory response may be secondary events of oxidant-induced lung injury.

O_3 also induces release of interleukin-6 and inhibits interleukin-2 release from lymphocytes [174, 175]; moreover, it inhibits mitogen-induced lymphocyte proliferation [175]. T-cell-dependent immunoglobin G (IgG) production also decreases after *in vitro* exposure of human lymphocytes to O_3 [174]. These results suggest that oxidant-induced changes in immunoregulation may be mediated by an altered T-cell function.

Tissue function

Functional alterations induced by oxidants have also been investigated on isolated tissues.

Using the isolated saline perfused rat lung model, PINO *et al.* [176] demonstrated that O_3 increases pulmonary resistance and decreases dynamic compliance. These functional alterations are accompanied by damage to airway epithelium and oedema, which resemble the lesions described after acute *in vivo* exposure, giving a further indication that inflammatory cells do not play a primary role in oxidant-induced lung injury.

In vitro airway smooth muscle responsiveness after *in vivo* exposure to oxidants has been studied to determine whether an alteration of the smooth muscle was involved in the effects exerted by these gases on lung

function. After exposure to O_3, preparations of airway smooth muscle develop *in vitro* an hyperresponsiveness to different stimuli, *i.e.* acetylcholine, electrical field stimulation, histamine and substance P, which activate smooth muscle through activation of their own specific receptors. By contrast, the response to KCl, which acts directly by depolarizing the plasma membrane, is not affected [177–179]. From these studies, it may be deduced that smooth muscle contractility is not altered, but that oxidants may affect airway responsiveness through alteration of receptor-mediated pathways. These studies have also suggested that the epithelium is involved in O_3-induced hyperresponsiveness and that small airways are more susceptible. We have recently studied the *in vitro* response of rat bronchial rings after exposure to NO_2 and we have found that this compound does not alter smooth muscle responsiveness [180, 181].

Conclusions

In conclusion, the studies reviewed in this article constitute a wide body of evidence about the adverse effects exerted on the respiratory system by the two most common pollutant oxidant gases. We have analysed the experimental data obtained from animal research, which allow some understanding of the mechanisms leading to health hazard. Once they have penetrated the airways, oxidant pollutants react with the epithelium lining fluid, possibly generating free radicals and other oxidant-diffusing products, which may then act on target cells. The main site of oxidant action is the terminal bronchiole, where epithelial cells and alveolar macrophages are damaged and produce mediators, leading to functional impairment, oedema and inflammation. Under these conditions, microbial infections are facilitated. Long-term exposure to oxidant gases may cause emphysema and/ or fibrosis, probably as a result of an altered metabolism of collagen and elastin. Throughout this cascade of events, a biochemical protective system is activated at cellular level, which may determine repair of the injury and development of tolerance to further exposures.

The relevance of insight from animal research to human health is an unresolved matter. Indeed, no animal species mimics humans in every aspect and a quantitative extrapolation to man of the effective pollutant concentration from animal studies is not appropriate. Nevertheless, several data from animal research have been taken into account when establishing air quality guidelines, mainly those obtained using long-term exposures and concentrations of gas that may presently be found in the environment. Indeed, results from experimental studies on humans (reviewed by SANDSTRÖM in this Series [182]) tally well with data from comparable experiments in animals. To emphasize the aspects which may be of greater relevance to man, we have summarized in table 1 the effects reported in animals after exposure to NO_2 and O_3 and the lowest gas concentrations which caused them.

With respect to the effects of ozone and nitrogen dioxide on the respiratory system, we would point out that: 1) high doses of both oxidants can cause death due to lung oedema; 2) sublethal short-term exposure to NO_2 and O_3 may cause reversible effects, such as altered lung function, airway hyperresponsiveness, epithelial damage, and inflammation; 3) long-term exposure may also cause irreversible damage, such as emphysema and pulmonary fibrosis; and 4) interactions either between NO_2 and O_3 or between one of them and other pollutants have synergistic effects.

The major impact of animal research is the understanding of the mechanisms of oxidant-induced respiratory injury, which may contribute to improve management and prevention of respiratory diseases associated with air pollution. Moreover, there is a continuous introduction of new pollutants into the atmosphere, which con-

Table 1. – Lower exposure concentrations of nitrogen dioxide and ozone which have been reported to cause adverse effects on the respiratory system in animals

Effect	Nitrogen dioxide			Ozone		
	ppm	Time	[Ref.]	ppm	Time	[Ref.]
Increased breathing frequency	0.8	#	[40]	0.34	2 h	[33]
Decreased tidal volume	5	2 h	[33]	0.34	2 h	[33]
Air trapping	0.2 (0.8)##	32 weeks	[42]	0.24	18 h	[34]
Airflow limitation	0.2 (0.8)##	32 weeks	[42]	0.26	2 h	[39]
Airway hyperresponsiveness	4	7 days	[54]	0.5	2 h	[38]
Damage to epithelium	2	2 years	[41]	0.06 (0.25)##	1 week	[75]
Lung oedema	0.4	1 week	[106]	0.06 (0.25)##	1 week	[75]
Inflammation	7	1 day	[66]	0.06 (0.25)##	1 week	[75]
Emphysematous lesions	1	6 months	[13]	-	-	-
Fibrotic lesions	1	6 months	[13]	0.06 (0.25)##	1 week	[75]
Reduced defence against infections	0.5	3 months	[137]	0.08	3 h	[136]

N.B. For both oxidants, the same effects have been demonstrated after exposure to higher concentrations for shorter times. #: This effect developed immediately and persisted with exposure, which lasted 2 yrs; ##: numbers are background exposure concentrations, while numbers between brackets are level of superimposed spike exposure. ppm: parts per million.

stitutes a potential risk for human health. Experimental animal studies may help to quantify and possibly to prevent them. Finally, the research on the mechanisms of pulmonary toxicity of oxidant gases may provide useful information about the mechanisms of lung injury in general, and, thus, they may contribute to the understanding of respiratory diseases such as asthma, bronchitis, pulmonary fibrosis and emphysema.

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