Ozone and nitrogen dioxide

A study on mechanisms of toxic action and cellular defense

Aan mijn ouders
Aan Sjef

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
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dr. C.C. Oosterlee,
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STELLINGEN

1. Ozon schade in levende organismen ontstaat via een ander mechanisme dan schade door stikstofdioxide.

   Dit proefschrift

2. De door glutathion peroxidase gekatalyseerde detoxificatie van vetzuur hydroperoxiden is niet van belang voor de glutathion - afhankelijke cellulaire afweer tegen ozon.

   - *C.K. Chow and A.L. Tappel. An enzymatic mechanism against lipid peroxidation damage to lungs of ozone-exposed rats. Lipids 7 (1972) 518-524.*
   - *Dit proefschrift*

3. De conclusie van Menzel et al. dat methyl ozonides glutathion oxideren tot zijn disulfide vorm is onvoldoende gefundeerd.

   D.B. Menzel, R.J. Slaughter, A.M. Bryant and H.O. Jauregui. Heinz bodies formed in erythrocytes by fatty acid ozonides and ozone. Arch. Environ. Health 30 (1975) 296-301.

4. Discussies omtrent additieven in de voeding zouden zich in het geval van de antioxidantia juist moeten richten op het risico van het niet toevoegen van deze stoffen.

5. Het is reëel te verwachten dat de isolatie van genen, relevant voor levensbedreigende ziekten, op langere termijn therapeutische voordelen oplevert die opwegen tegen mogelijke nadelen van de genetische screening.

   G. Kolata. Genetic screening raises questions for employers and insurers. Science 232 (1986) 317-319.

6. Het interdisciplinaire karakter van de toxicologie rechtvaardigt een afzonderlijke universitaire opleiding.

7. De conclusie van Rasmussen dat het door Alink et al. ontwikkelde in vitro expositie model voor O_3 en NO_2 slechts enkele toepassingen zou kennen duidt op zijn gebrek aan ervaring met dit model systeem.

   - *G.M. Alink, J.C.M. van der Hoeven, F.M.H. Debets, W.S.M. van de Ven, J.H. Koeman and J.S.M. Boleij. A new exposure model for in vitro testing of effects of gaseous pollutants on mammalian cells by means of gas diffusion through plastic films. Chemosphere 2 (1979) 63-73.*
   - *R.E. Rasmussen. In vitro systems for exposure of lung cells to NO_2 and O_3. J. Toxicol. Environ. Health, 13 (1984) 397-411.*

8. Ondanks het feit dat al bijna 30 jaar bekend is dat bijvoorbeeld polycyclische aromaten stereoselectief worden gemetaboliseerd, wordt met dit fenomeen nog steeds te weinig rekening gehouden bij de bestudering van de relatie tussen toxiciteit en biotransformatie.

   - *R.T. Williams. Detoxication mechanisms. Chapman and Hall Ltd. London 1959, p. 227.*
   - *E.J. Ariens. Chirality in bioactive agents and its pitfalls. Trends in Pharmacological Sciences 7 (1986) 200-205.*

9. Nieuwe mogelijkheden van de hoge-temperatuur materiaaltechnologie dienen meer te worden benut voor het terugdringen van de beperking gegeven door het Carnot rendement.

10. Het ontbreken van een volledig representatief diermodel voor de ziekte van Alzheimer is mogelijk een weerspiegeling van het feit dat bij deze ziekte evolutionair gezien laat verworven eigenschappen van hogere intelligentie worden aangetast.

11. De verontrusting op het gebied van de bodemverontreiniging neemt toe met het afnemen van de detectiegrenzen.

Stellingen, behorende bij de proefschrift "Ozone and nitrogen dioxide. A study on mechanisms of toxic action and cellular defense." Ivonne M.C.M. Rietjens. Wageningen 19 december 1986.

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CONTENTS

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Ivonne M.C.M. Rietjens

PART I

INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

1.1. Historical backgrounds

According to an old review by Andrews (1874) ozone was discovered in the last century by Schönbein and described for the first time in 1840. The toxic action of ozone was already observed in the nineteenth century by Redfern and by Dewar and McKendrick who demonstrated ozone to be lethal to experimental animals (Andrews, 1874).

In the years before 1920 early investigators demonstrated the lung and respiratory tract to be the primary target of ozone (Schulz, 1892; Hill and Flack, 1912; Jordan and Carlson, 1913).

Attention to the problem of ozone and nitrogen dioxide toxicity especially increased when ozone was shown to be a key component of photochemical smog, arising from the reactions between hydrocarbons, sunlight (UV) and nitrogen dioxide, and also when ozone was found to be present in airoplane cabins at high altitudes and to arise from several industrial processes such as inert-gas shielded arc welding, air and water purification using ozonizing equipment, and high voltage electric processes (Stokinger, 1965).

Effects of ozone on man exposed under experimental or occupational conditions, were reported first in the nineteen fifties and included dryness of mouth and throat, respiratory tract irritation, continuous headache, shortness of breath and pulmonary congestion (Wilska, 1951; Truche, 1951; Kleinfeld and Giel, 1956). Because the toxicity of nitrous fumes was also well recognized at this time, the question arose whether the toxic responses observed in these studies could arise from oxides of nitrogen as well (Kleinfeld and Giel, 1956; Ferry and Ginther, 1952).

During the late fifties and early sixties experiments with human volunteers, exposed under controlled conditions, revealed information

on the effects of ozone on several pulmonary functional parameters such as vital capacity, 0.75-sec forced expiratory volume and maximal midexpiratory flow rate (Griswold et al., 1957; Young et al., 1964). In the same period effects of ozone on a number of species of experimental animals were also studied in more detail. Histological, physiological and biochemical changes in experimental animal lung upon acute and chronic exposure were reported, as well as many factors influencing ozone toxicity to experimental animals. These studies are reviewed by Stokinger (1965).

Two aspects of ozone toxicity, i.e. acute toxic pulmonary edema and the phenomenon of tolerance development, directed the attention of investigators to the molecular mechanisms by which ozone caused these toxic effects.

Ozone and nitrogen dioxide appeared to oxidize a range of model compounds (unsaturated hydrocarbons, unsaturated fatty acids, amino acids, sulfhydryl compounds and proteins) when these were exposed under a-biotic conditions. (Mudd et al., 1969; Roehm et al., 1971a; Roehm et al., 1971b; Ramazzotto et al., 1971; Felmeister et al., 1970; Stedman et al., 1973; Pryor et al., 1976; Srisankar and Patterson, 1979).

At present such in vitro studies in which cell components or model compounds are exposed, still form an important basis for the study of the molecular reactions of ozone and nitrogen dioxide, and provide information on different possibilities for their mode of toxic action (Pryor and Lightsey, 1981; Pryor et al., 1982).

However, up to now, no conclusive evidence on the mechanism of toxic action in an intact cell system is available. Some laboratories have reported increased amounts of products of lipid peroxidation in the lungs of exposed animals (Thomas et al., 1968; Goldstein et al., 1969; Sagai et al., 1982) although other laboratories have been unable to confirm these results (Mustafa and Tierney, 1978). Contradictory results were also reported for the measurement of other products of lipid peroxidation (ethane and pentane) in the breath of exposed

animals (Dumelin et al., 1978a; Dumelin et al., 1978b; Dillard et al., 1980; Tappel and Dillard, 1981; Sagai et al., 1981; Sagai et al., 1982). The only unequivocal evidence for the involvement of an oxidative reaction pathway in the toxic action of ozone or nitrogen dioxide is of an indirect nature, as it is provided by the protection of exposed animals by antioxidant compounds such as vitamin E and C (Matzen, 1957; Fletcher and Tappel, 1973; Donovan et al., 1977; Chow et al., 1979; Kratzing and Willis, 1980; Chow et al., 1981).

The development of **i)** techniques for cell culture and isolation of primary lung cell cultures, and of **ii)** model systems for the exposure of cells in vitro to oxidative gaseous compounds, during the last two decades, offers a new approach for studying the mechanisms of toxic action of oxidant gases in intact cell systems. An advantage of the cell model is that more or less homogeneous cell populations can be studied, although it is recognized that effects found in isolated cell cultures always need in vivo validation.

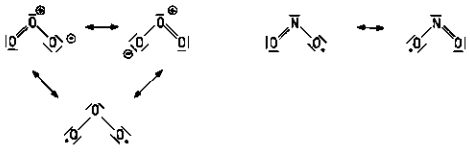
The experiments described in this thesis were carried out with primary cultures of lung cells isolated from rats as well as cell lines originating from human lung tissue (A549), and a model system for in vitro exposure of cell cultures to gaseous compounds which was developed at our laboratory (Alink et al., 1979). Exposure of both control or biochemically modified cell cultures offered the possibility for studying both the mode of action of the compounds concerned as well as possible mechanisms of cellular defense, in an intact cell model instead of in an a-biological chemical model system.

1.2. Physical and chemical constants

Table 1 summarizes the main physical and chemical properties of ozone and nitrogen dioxide.

It is noteworthy to recognize that ozone has a biradical or ionair nature, whereas nitrogen dioxide is a free radical. These characteristics may be of importance with regard to the reactions with cell components.

TABLE 1. PHYSICAL AND CHEMICAL CHARACTERISTICS OF OZONE (O_3) AND NITROGEN DIOXIDE (NO_2). (Merck Index, 1976; Handbook of Chem. Phys., 1980).

Parameter	O_3	NO_2
* molecular structure :		
* molecular weight :	48	46
* boiling point (1 atm) :	- 111.9 °C	21.15 °C
* melting point (1 atm) :	- 192.7 °C	- 9.3 °C
* density gas (1 atm) :	2.144 g/l (0°C)	3.3 g/l (21.3 °C)
* density liquid (1 atm):	1.614 g/ml (-195.4°C)	1.448 g/ml (20 °C, 4 atm)
* critical temperature :	- 12.1 °C	158.2 °C
* critical pressure :	53.8 atm	99.96 atm
* conversion factors:		
1 ppm :	2000 μ g/m^3	1880 μ g/m^3
1 mg/m^3:	0.500 ppm	0.532 ppm

1.3. Natural and man made sources

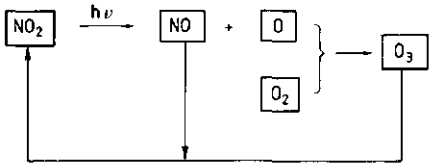
natural background levels

Natural background levels of ozone in the lower atmosphere (10-80 μ g/m^3 = 0.005-0.04 ppm) (Fabian and Pruchniewicz, 1973) arise from atmospheric circulation, transporting ozone formed in the stratosphere by photolysis of molecular oxygen, to the lower atmosphere, from photochemical oxidation of natural hydrocarbons, including terpenes

from trees and other vegetation, and it is formed in the atmosphere by lightning, originating from oxygen. (WHO, 1979).
Natural background levels of nitrogen dioxide (0.4-9.4 $\mu g/m^3$ = 0.0002-0.005 ppm) (WHO, 1977) mainly come from lightning, vulcanic eruptions and bacterial processes in the soil (WHO, 1977).

Outdoor concentrations

Various man-made sources cause the ozone and nitrogen dioxide concentrations to rise above natural background levels. Nitric oxide (NO), emitted after combustion of fossil fuels (e.g. traffic, industrial processes), is the major source of nitrogen dioxide, as it is readily oxidized to nitrogen dioxide in the atmosphere (WHO, 1977).
The main ozone producing process in the lower atmosphere, identified sofar, is depicted in Figure 1. The overall principal is that solar radiation interacts with nitrogen dioxide to produce ozone and nitric



$$NO_2 + h\nu\ (\lambda < 430nm) \xrightarrow{k_1} NO + O \qquad 1$$
$$O + O_2 + M \xrightarrow{k_2} O_3 + M \qquad 2$$
$$NO_2 + O_2 \xrightarrow{h\nu} NO + O_3 \qquad 1+2$$
$$NO + O_3 \xrightarrow{k_3} NO_2 + O_2 \qquad 3$$

Figure 1: Interrelationship between atmospheric concentrations of nitric oxide (NO), nitrogen dioxide (NO_2) and ozone (O_3).
$h\nu$: represents solar radiation.
M :an energy rich molecule, usually O_2 or N_2 necessary for the reaction to take place.
(Becker and Schurath, 1975).

oxide. The nitric oxide can in turn react with ozone to give oxygen and nitrogen dioxide again (Becker and Schurath, 1975; WHO, 1979). At equilibrium, this series of chemical reactions results in an ozone concentration in the lower atmosphere that is determined by the following equation:

$$[O_3] = \frac{k_1 \cdot [NO_2]}{k_3 \cdot [NO]}$$

with k_1 dependent on light intensity. From this equation it can be derived that at night-time continuous nitric oxide emissions will cause a rapid reduction of the ozone concentration, due to reaction 3 (Figure 1), whereas during day-light reaction 1 will cause the equilibrium to shift in favor of ozone production.

Several measurements of diurnal concentrations of ozone and nitrogen dioxide have confirmed the above mentioned model (Derwent and Stewart, 1973; Becker and Schurath, 1975).

In addition to these processes the presence of organic hydrocarbon compounds in the polluted atmosphere may give rise to a great number of reactions ultimately resulting in an increase in the concentration of ozone (Becker and Schurath, 1975).

Due to all these processes ozone concentrations in the lower atmosphere may rise to maximum one-hour values amounting to 200-800 $\mu g/m^3$ (0.10-0.40 ppm) (WHO, 1979). In Los Angeles a one-hour value of even 0.96 ppm O_3 (1920 $\mu g\ O_3/m^3$) has been reported (Goldstein, 1977).

Nitrogen dioxide concentrations may rise to maximum one-hour values as high as 100-800 $\mu g/m^3$ (0.05-0.43 ppm) and to maximum 24-hour mean values of 100-400 $\mu g/m^3$ (0.05-0.22 ppm).

Table 2 gives an example of the total emissions of oxides of nitrogen and hydrocarbons, which are responsible for the formation of ozone.

TABLE 2. EMISSIONS OF OXIDES OF NITROGEN (NO_x) AND HYDROCARBONS IN THE NETHERLANDS, FROM DIFFERENT SOURCES (in million kg/year) (C.B.S. 1986).

source	NO_x 1980	NO_x 1982	hydrocarbons 1980	hydrocarbons 1982
* process emissions (all industrial processes except combustion of fossil fuels)	30	29	206	187
* combustion of fossil fuels (industries and housekeepings)	194	187	28	25
* traffic	300	300	215	190
* others	-	-	90	90

Indoor concentrations

For ozone, indoor levels tend to be lower than those outside, but the use of for example ultraviolet lamps, photocopying machines or certain industrial technological processes such as hydrogen peroxide production or inert-gas-shielded arc welding, occasionally give rise to increased indoor exposure (Kleinfeld and Giel,1956; Stokinger, 1965). In addition, relative high concentrations of ozone can be detected in airoplanes (Stokinger, 1965; Lategola et al., 1980).

Generally the maximum indoor ozone concentrations reached in all these situations are in the same order of magnitude as the concentrations found outdoors, i.e. 200-2000 $\mu g/m^3$ (0.1-1.0 ppm) (WHO, 1979).

As compared to ozone, the exposure of people to nitrogen dioxide by sources other than the outdoor environment, is usually underestimated. Nitrogen dioxide formation by gas-fired domestic appliances may give rise to concentrations up to 2000 $\mu g/m^3$ (1.1 ppm) (WHO, 1977), and tobacco smoke was reported to contain nitrogen dioxide levels of 19-95 mg/m^3 (10-50 ppm) (WHO, 1977).

Obviously these indoor sources can contribute substantially to the daily nitrogen dioxide exposure of people.

1.4. Toxic effects of ozone and nitrogen dioxide

Animal data

Excellent reviews concerning the toxic effects of ozone and nitrogen dioxide on experimental animals are available in the literature (WHO, 1977; Mustafa and Tierney, 1978; WHO, 1979; Menzel, 1984; Morrow, 1984). Therefore only a brief review will be presented in this thesis. In general, both gases are known to cause comparable toxic effects on morphological, cytodynamical, functional and biochemical lung parameters. Examples of the main effects of ozone and nitrogen dioxide on experimental animals are given in Table 3.

The proliferation of type II alveolar pneumocytes and of bronchiolar non-ciliated Clara cells reported for both ozone and nitrogen dioxide exposed animal lungs (Table 3), is generally believed to occur after damage to the more sensitive alveolar type I pneumocytes and ciliated bronchiolar epithelial cells, respectively (Evans, 1984).

TABLE 3. MAIN EFFECTS OF OZONE AND NITROGEN DIOXIDE EXPOSURE ON EXPERIMENTAL ANIMALS.

Effects	Exposure conditions	References
MORPHOLOGICAL CHANGES		
* degeneration and destruction of type I alveolar pneumocytes	* 0.5-3 ppm O_3 several hours * 2-18 ppm NO_2 several days	* Plopper et al. 1973 Stephens et al. 1974 * Evans et al. 1972 Stephens et al. 1972 Evans et al. 1978

* loss of ciliated epithelium from the upper airway	* 0.2-0.8 ppm O_3 several days or 0.3-3 ppm O_3 several hours	* Plopper et al. 1973 Bils 1974 Boatman et al. 1974 Evans et al. 1976 Schwartz et al. 1976 Ibrahim et al. 1980
	* 2-30 ppm NO_2 several days	* Stephens et al. 1972 Bils 1974 Evans et al. 1976
* oedematous changes of alveolar epithelial cells	* 1-12 ppm O_3 several hours	* Alpert and Lewis 1971 Plopper et al. 1973
	* 10-100 ppm NO_2 several hours	* Hine et al. 1970 Stephens et al. 1972 WHO, 1977
LUNG CYTODYNAMICS		
* proliferation of type I alveolar pneumocytes	* 0.2-0.8 ppm O_3 several days	* Schwartz et al. 1976 Eustis et al. 1981
	* 12-18 ppm NO_2 several days	* Stephens et al. 1972 Evans et al. 1972 Evans et al. 1978
* proliferation of bronchiolar nonciliated Clara cells	* 0.2-0.8 ppm O_3 several days	* Schwartz et al. 1976 Evans et al. 1976 Ibrahim et al. 1980
	* 15-17 ppm NO_2 24 hours	* Evans et al. 1976
* increase in the number of alveolar macrophages	* 0.1-0.8 ppm O_3 several days	* Schwartz et al. 1976 Zitnik et al. 1978 Chow et al. 1981 Eustis et al. 1981
	* 12-18 ppm NO_2 several days	* Evans et al. 1972 Stephens et al. 1972 Evans et al. 1978

FUNCTIONAL PARAMETERS		
* impairment of the function of alveolar macrophages	* 0.2-9 ppm O_3 several hours * 2.3-15 ppm NO_2 several hours	* Coffin et al. 1968 Goldstein et al. 1971 * Goldstein et al. 1973
* increased susceptibility to inhaled infectious agents	* 3-10 ppm O_3 several hours * 2.3-15 ppm NO_2 several hours	* Purvis et al. 1961 Coffin et al. 1968 Goldstein et al. 1971 * Goldstein et al. 1973
* increased flow resistance	* 0.3-0.5 ppm O_3 several hours	* Watanabe et al. 1973
* increased respiratory frequency and decreased tidal volume	* 0.3-1.4 ppm O_3 several hours * 5.2-13 ppm NO_2 several hours	* Murphy et al. 1964 * Murphy et al. 1964
BIOCHEMICAL PARAMETERS		
* increase in the activity of lung antioxidant enzymes (glutathione peroxidase, glutathione reductase, glucose-6-P dehydrogenase)	* 0.1-1 ppm O_3 several days * 2.3-10 ppm NO_2 several days	* Chow and Tappel 1973 Chow et al. 1974 Chow et al. 1981 * Chow et al. 1974 Sagai et al. 1981 Sagai et al. 1982
* decrease in lung content of reduced glutathione	* 2-4 ppm O_3 several hours * 0.8 ppm NO_2 several days	* DeLucia et al. 1972 DeLucia et al. 1975 * Nakajima and Kusumoto 1968
CHRONIC EFFECTS		
* lung fibrosis	* 0.5-3 ppm O_3 several weeks or months	* Freeman et al. 1973 Last et al. 1979 Hesterberg and Last 1981

* lung emphysema	* 0.4-0.9 ppm O_3 several weeks or several months	* P'an et al. 1972 Freeman et al. 1974
	* 0.5-15 ppm NO_2 several months	* Blair 1969 Freeman et al. 1974
EXTRA PULMONARY EFFECTS		
* effects on blood		
- Heinz body formation in red blood cells	* 0.85 ppm O_3 4 hours	* Menzel et al. 1975
- increased fragility of red blood cells	* 8 ppm O_3 4 hours	* Goldstein et al. 1968
- aminotransferase activity increased	* 1 ppm NO_2 several months	* Kosmider and Misiewicz 1973
- red blood cell acetyl-cholinesterase activity reduced	* 8 ppm O_3 4 hours	* Goldstein 1968
* effects on liver		
- increased ascorbic acid levels	* 0.2-0.4 ppm O_3 several hours	* Veninga and Lemstra 1975
	* 0.3-4 ppm NO_2 several hours	* Veninga and Lemstra 1975
- decreased aminotransferase activity	* 1 ppm NO_2 several months	* Kosmider and Misiewicz 1973
* effects on reproduction		
- increased neonatal mortality	* 0.1-0.2 ppm O_3 3 weeks	* Brinkman et al. 1964

Effects on man

The effects of ozone and nitrogen dioxide on man described in the literature are mainly based on studies with human volunteers exposed under controlled conditions. Table 4 summarizes examples of the main effects reported.

Epidemiological studies of exposed populations are only scarcely available and always encounter the problem of whether the observed health effects can be really attributed to ozone or nitrogen dioxide or have to be ascribed to other components in the polluted atmosphere. This, because photochemical smog is known to consist of peroxy acyl-nitrates (PAN), other nitrate compounds, sulfates and reducing agents such as sulfur dioxide as well.

TABLE 4. EFFECTS OF OZONE AND NITROGEN DIOXIDE ON HUMAN VOLUNTEERS EXPOSED UNDER CONTROLLED CONDITIONS.

Effects	Exposure conditions	References
SENSORY EFFECTS		
* odour perception	* 0.008-0.05 ppm O_3	* Henschler et al. 1960 WHO 1979
	* 0.12-0.42 ppm NO_2	* Henschler et al. 1960 WHO 1977
* respiratory tract irritation, dry cough, chest discomfort	* 0.35-0.75 ppm O_3 several hours	* Kerr et al. 1975 Silverman et al. 1976
EFFECTS ON RESPIRATORY FUNCTION		
* changes in pulmonary function (increase in airway resistance, reduction in forced respiratory volume)	* 0.35-0.75 ppm O_3 several hours	* Kerr et al. 1975 Silverman et al. 1976 Golden et al. 1978 Hackney et al. 1978
	* 5-30 ppm NO_2 several minutes or 1 hour	* von Nieding et al. 1973

EXTRAPULMONARY EFFECTS		
* effects on blood		
- increased red blood cell fragility	* 0.5 ppm O_3 several hours	* Buckley et al. 1975
- increased glucose-6-P dehydrogenase and lactate dehydrogenase activity in red blood cells	"	"
- decreased red blood cell acetylcholinesterase activity and glutathione level	"	"
- increased serum vitamin E	"	"
- decreased serum glutathione reductase activity	"	"

Mutagenic, clastogenic and carcinogenic effects

Only few experimental data are available on the possible mutagenic and carcinogenic effects of ozone and nitrogen dioxide. For ozone, they are summarized in Table 5.

The data summarized in Table 5 indicate that ozone might be a mutagenic agent, but the relevance of these in vitro studies to in vivo exposure is doubtful, because there are conflicting results from both human and animal studies. Guerrero et al., (1979), for example, reported sister chromatid exchanges (SCE's) in WI-38 cells exposed to ozone, but exposure of human volunteers for 2 hours to 0.5 ppm ozone produced no elevation of SCE's in peripheral lymphocytes (Guerrero et al. 1979). Besides McKenzie et al. (1977) were unable to show an increase in chromosomal damage in the circulating lymphocytes of 30

volunteers exposed for 4 hours to 0.4 ppm ozone, while results reported by Merz et al. (1975) demonstrated chromosome aberrations in lymphocytes of human subjects exposed to 0.5 ppm ozone for 6 or 10 hours.

TABLE 5. MUTAGENIC, CLASTOGENIC AND CARCINOGENIC EFFECTS INDUCED BY OZONE.

Effects	References
CLASTOGENIC EFFECTS	
* chromosomal abnormalities, i.e. chromosome breakages and chromatid deletions in human and animal cells exposed *in vitro* or *in vivo*	Fetner 1962 Zelac et al. 1971 Merz et al. 1975 Dubeau and Chung 1979
* sister chromatid exchanges in WI-38 cells	Guerrero et al. 1979
MUTAGENIC EFFECTS	
* base substitutions and frame-shift mutations in *Saccharomyces cerevisiae*	Dubeau and Chung 1982
* forward mutations in different strains of *Escherichia coli*	L'Herault and Chung 1984
* forward mutation on the HGPRT locus of V79 cells	Chu 1971
CARCINOGENIC EFFECTS	
* increased tumor incidence in mice exposed to ozonized gasoline for 52-92 weeks	Kotin and Falk 1956 Kotin et al. 1958
* increase in lung tumor number in mice after exposure to 0.3-0.5 ppm O_3 intermittently for 6 months	Hassett et al. 1985

With respect to the carcinogenic and mutagenic properties of nitrogen dioxide the possible formation of nitrosamines by the reaction of nitrite, arising from the reaction of nitrogen dioxide with water (section 2.1), with tissue amines should be considered (von Nieding, 1978; Pryor and Lightsey, 1981; Morrow, 1984). As far as we know, no evidence is present concerning the mutagenicity or carcinogenicity of nitrogen dioxide itself.

Obviously mutagenic and carcinogenic effects of ozone and nitrogen dioxide should be investigated in more detail before definite conclusions can be drawn.

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CHAPTER 2

THEORIES ON THE MODE OF TOXIC ACTION AND CELLULAR PROTECTION

The general idea on the mode of toxic action of ozone and nitrogen dioxide is that they exert their toxic effect by means of their oxidative capacity. Oxidation of the unsaturated fatty acids in the membrane phospholipids (section 2.1) and/or of thiol groups and sensitive amino acid residues of structural and functional proteins (section 2.2) offers a likely common mechanism of toxic action (Menzel, 1984; Morrow, 1984). However, it is still unknown whether the cellular reaction mechanisms underlying these oxidations are the same for nitrogen dioxide and ozone, or not.

2.1. Oxidation of unsaturated fatty acid moieties of membrane phospholipids

With respect to the oxidation of the unsaturated fatty acids in the membrane phospholipids, two reaction mechanisms have been proposed in the literature, either initiated by abstraction of an allylic hydrogen atom or by addition of the ozone or nitrogen dioxide molecule to the double bond of the unsaturated fatty acid. Both theories are mainly based on in vitro studies with pure fatty acid monolayers or suspensions of pure fatty acids.

The abstraction mechanism

This reaction mechanism suggested for the oxidation of unsaturated fatty acid membrane moieties appears to be the same for both ozone and nitrogen dioxide. The mechanism is depicted in Figure 1. It is initiated by the abstraction of an allylic hydrogen atom from the unsaturated fatty acid molecule (Pryor and Lightsey, 1981; Pryor et al., 1981; Pryor et al., 1982; Pryor et al., 1983).

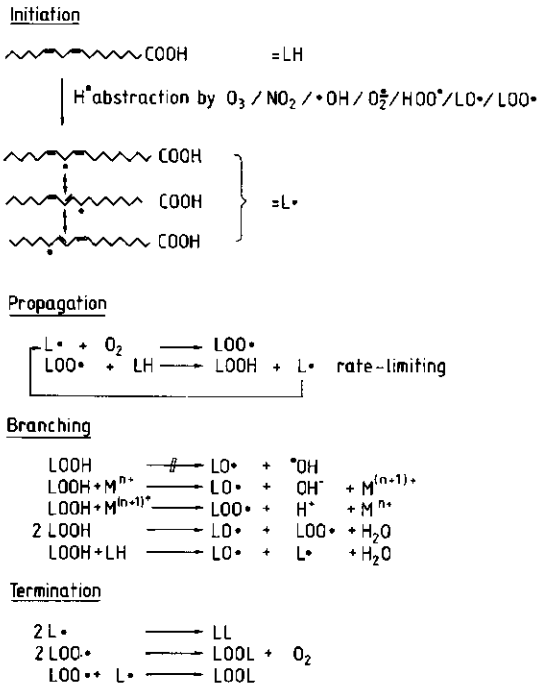

Figure 1. Reaction scheme of radical mediated lipid peroxidation, initiated by hydrogen abstraction by ozone (O_3), nitrogen dioxide (NO_2) or reactive species possibly arising from these compounds.

In addition to initiation of this lipid peroxidative pathway by hydrogen abstraction by ozone or nitrogen dioxide themselves, hydrogen abstraction could possibly be achieved by initial reactive species resulting from the reaction of ozone or nitrogen dioxide with water. Alder and Hill (1950) and Sachsenmaier et al. (1965), reported the formation of hydroperoxyl (HOO•) and hydroxyl (•OH) free radicals from the decomposition of ozone in aqueous media, following the reaction sequence:

24.

$$O_3 + H_2O \longrightarrow HO_3^+ + OH^-$$
$$HO_3^+ + OH^- \rightleftharpoons 2\,HOO^\bullet$$
$$O_3 + HOO^\bullet \longrightarrow {}^\bullet OH + 2\,O_2$$
$${}^\bullet OH + HOO^\bullet \longrightarrow H_2O + O_2$$

Dismutation of the hydroperoxyl free radical follows from a reaction with another hydroperoxyl free radical or with its deprotonated form, the superoxide free radical ($O_2^{\bullet-}$) readily formed from $HOO^{\bullet}$ at neutral pH where the concentration ratio $O_2^{\bullet-}/HOO^{\bullet}$ is 400 (Fukuzawa and Gebicki, 1983).

Dismutation of the hydroperoxyl free radical proceeds by the following reactions and gives rise to the formation of hydrogen peroxide:

$$HOO^{\bullet} + H_2O \rightleftharpoons O_2^{\bullet-} + H_3O^+$$

$$HOO^{\bullet} + O_2^{\bullet-} + H_3O^+ \longrightarrow H_2O_2 + O_2 + H_2O$$

$$HOO^{\bullet} + HOO^{\bullet} \longrightarrow H_2O_2 + O_2$$

Generation of hydrogen peroxide by ozone in aqueous medium has also been suggested by other reports in the literature (Wenzel and Morgan, 1982; Wenzel and Morgan, 1983).

Finally, simultaneous production of $O_2^{\bullet-}$ and H_2O_2 is an additional source of hydroxyl free radicals, produced in the iron catalyzed Haber-Weiss reaction (Haber and Weiss, 1934; Bus and Gibson, 1979; Winterbourn, 1981; Winterbourn, 1982).

$$O_2^{\bullet-} + Fe^{3+} \longrightarrow {}^1O_2 + Fe^{2+}$$

$$+ \quad Fe^{2+} + H_2O_2 \longrightarrow OH^{\bullet} + OH^- + Fe^{3+}$$

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{Fe^{3+}/Fe^{2+}} OH^{\bullet} + OH^- + {}^1O_2$$

The oxygen molecule formed in this reaction may be the reactive electronically excited form of O_2, singlet oxygen (= $^1O_2$) (Kellog and Fridovich, 1975; Koppenol, 1976).

In addition, $O_2^{\bullet-}$ and $^{\bullet}OH$ can also give rise to the production of singlet oxygen in the reactions (Koppenol, 1976; Bus and Gibson, 1979; Singh, 1982):

$$2\,O_2^{\bullet-} + 2\,H_2O \longrightarrow H_2O_2 + 2OH^{\bullet} + {}^1O_2$$

$$^{\bullet}OH + O_2^{\bullet-} \longrightarrow OH^- + {}^1O_2$$

The main reaction products formed upon the reaction of nitrogen dioxide with water are the reactive hydroxyl free radical and nitrite. They arise from the reaction (Huygen and Lanting, 1975):

$$NO_2 + H_2O \longrightarrow {}^{\bullet}OH + HNO_2$$

Depending on the reaction conditions this reaction can be followed by

$$NO_2 + {}^{\bullet}OH \longrightarrow HNO_3$$

resulting in the overall reaction (Huygen and Lanting, 1975):

$$2\,NO_2 + H_2O \longrightarrow HNO_2 + HNO_3$$

The initiation of lipid peroxidation by hydrogen abstraction, achieved by ozone or nitrogen dioxide themselves or by one of these reactive oxygen species formed upon the reaction of the gaseous compounds with water, is followed by a propagative chain reaction. Propagation of the lipid peroxidative reaction sequence is achieved by addition of an oxygen molecule to the lipid free radical (L$^{\bullet}$), followed by a subsequent hydrogen abstraction by the lipid peroxyl free radical (LOO$^{\bullet}$), which gives rise to a lipid hydroperoxide (LOOH) and again a lipid free radical (L$^{\bullet}$) capable of entering the same reaction cycle (Figure 1) (Mead, 1976; Holman, 1954).

In this way a chain reaction is initiated, of which the slowest, and therefore rate limiting reaction is the hydrogen abstraction from a subsequent fatty acid molecule (Witting, 1970).

This hydrogen abstraction occurring either as the initiation reaction or in the propagation sequence, also is the only reaction step dependent on the structural characteristics of the unsaturated double bond(s) in the fatty acid molecule (Witting, 1970). Holman (1954) has demonstrated that the relative rate of autoxidation increases according to the ratios of approximately 0.025:1:2.5:5:?:7.5, with the number of unsaturated fatty acid increasing from 1 to 6.

Branching of the chain reaction occurs when the lipid hydroperoxides arising from this reaction sequence decompose into reactive chain-initiating radicals following the equations given in Figure 1 (Gardner et al., 1974; Pryor, 1976; Mead, 1976).
Based on the bond dissociation energy of the O–O bond in tertiair-butyl-hydroperoxide, Pryor (1976) calculated that the rate constant for the unimolecular decomposition of this hydroperoxide:

$$LOOH \longrightarrow LO^{\bullet} + {}^{\bullet}OH$$

is about 5×10^{-16} sec^{-1} at 37°C, giving a half life time of 10^9 years. From this is concluded that such an uncatalyzed unimolecular decomposition is not likely to occur in a cellular system, whereas the metal catalyzed or molecule assisted decomposition of lipid hydroperoxides may give rise to new free radical species (Figure 1) (Pryor, 1976; Bus and Gibson, 1979).
Termination of the lipid peroxidative chain reaction can take place by cross-linking of membrane phospholipids (Figure 1), but it can also be achieved by the action of cellular antioxidant systems, which are to be discussed in section 1.6.

The addition mechanism

The second theory proposed for ozone or nitrogen dioxide induced oxidation of unsaturated fatty acids is also mainly based on <u>in vitro</u>

exposures of fatty acid monolayers or suspensions, and has not yet been demonstrated to occur in vivo.

The theory suggests that the oxidation is initiated by an addition of the gaseous compound to the double carbon-carbon bond of the unsaturated lipid molecules. However, in spite of this apparently corresponding initial reaction, the oxidative pathway following this addition is different for nitrogen dioxide and ozone respectively.

Addition of nitrogen dioxide to the double bond of an unsaturated fatty acid results in direct formation of a lipid radical which might subsequently give rise to a peroxidative process comparable to the one described above (Figure 2). So in general lipid oxidation induced by nitrogen dioxide proceeds by a radical mediated peroxidative reaction scheme, when induced by the hydrogen abstraction or the nitrogen dioxide addition mechanism.

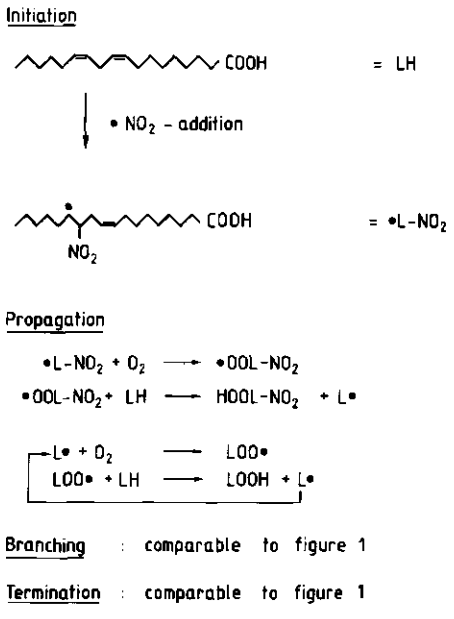

Figure 2. Reaction scheme for radical mediated lipid peroxidation initiated by nitrogen dioxide (NO_2) addition.

Based on experimental data presented by Pryor and Lightsey (1981) and Pryor et al. (1982) it appears that the initiation reaction of nitrogen dioxide induced oxidation of unsaturated carbon-carbon bonds, changes from predominantly addition at extremely high concentrations of nitrogen dioxide (> 1% = 10000 ppm) to predominantly abstraction at relatively low ppm levels of nitrogen dioxide (<100 ppm). This change in the initiation mechanism from addition to abstraction may be caused by the competition between a reversible addition reaction, favoured only at high concentrations of nitrogen dioxide, and an irreversible hydrogen abstraction step (Pryor and Lightsey, 1981; Pryor et al., 1982).

Pryor et al. (1982) demonstrated that at nitrogen dioxide concentrations below 100 ppm oxidation of fatty acid model compounds, i.e. methyl oleate (18:1), methyl linoleate (18:2) or methyl linolenate (18:3), is initiated for at least 75-90% by abstraction of an allylic hydrogen atom.

On the basis of these data it can be assumed that when air containing nitrogen dioxide is inhaled, polyunsaturated fatty acids in the lung react with nitrogen dioxide by a mechanism initiated by hydrogen abstraction, the addition mechanism being highly unlikely.

Finally it is of importance to notice that abstraction of an hydrogen atom by nitrogen dioxide gives rise to nitrous acid which might react with amines to produce nitrosamines (Pryor et al., 1982)(see section 1.3).

In contrast to the addition of the nitrogen dioxide free radical to a double carbon-carbon lipid bond, the addition of ozone does not result in direct formation of a lipid free radical. The reaction pathway following addition of ozone to an unsaturated lipid bond may proceed as suggested by Criegee (1957) (Figure 3). The mechanism appears to proceed by means of ionair instead of free radical initial intermediates. Oxidation of membrane lipids by this ionair mechanism may eventually result in formation of ozonides, aldehydes and lipid hydro-

peroxides (Figure 3). It is postulated in the literature, that these intermediates are involved in the toxic action of ozone, because they might for instance decompose into free radicals, which - in turn - could give rise to a radical mediated lipid peroxidation (Pryor et al., 1976). This implies that the oxidation of unsaturated fatty acids by ozone, when initiated by means of the ionair addition mechanism, may still proceed by a radical mediated mechanism of toxic action.

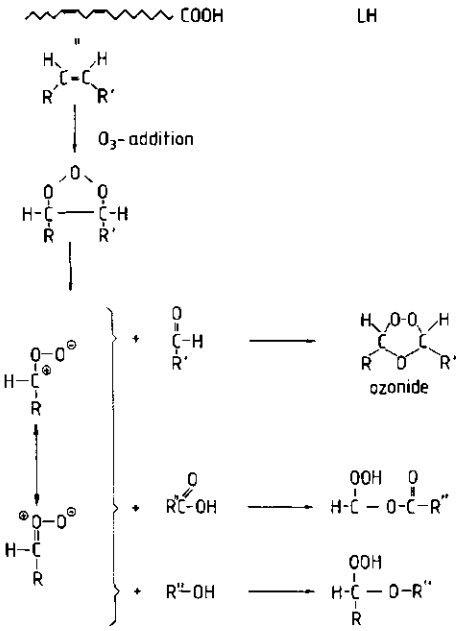

Figure 3. The ionair Criegee reaction mechanism for oxidation of unsaturated fatty acids initiated by ozone (O_3) addition.

Studies using pure fatty acids (Goldstein et al., 1968b; Roehm et al., 1971a; Roehm et al., 1971b; Pryor et al., 1976; Srisankar and Patterson, 1979), or aqueous emulsions of fatty acids (Roehm et al., 1971a; Roehm et al., 1971b) have demonstrated the ability of ozone to react by either one of the reaction mechanisms. The influence of the

oxidant concentration on the reaction mechanism followed has not been studied as extensively as for nitrogen dioxide, but even at low ppm concentrations of ozone, i.e. 1.5 ppm (Roehm et al., 1971a; Roehm et al., 1971b) and 0.03 ppm (Srisankar and Patterson, 1979) the oxidation of the unsaturated fatty acid model compounds seemed to proceed predominantly by the addition mechanism.
Based on data from experiments in which fatty acid model compounds were exposed to 0.08-1.5 ppm ozone, Pryor et al. (1976) even suggested that some initial product produced, perhaps an ozonide, might react to produce radicals which could initiate lipid peroxidation. Obviously, this hypothesis combines both possible mechanisms of toxic action known for ozone.

The most convincing evidence for the involvement of a radical mediated lipid peroxidation in the mechanism of toxic action of both ozone and nitrogen dioxide can be found in the protection provided by the phenolic antioxidant vitamin E (= α-tocopherol) (Thomas et al., 1968; Fletcher and Tappel, 1973; Chow et al., 1979; Chow et al., 1981) and also in the increased amount of ethane or pentane detected in the breath of ozone or nitrogen dioxide exposed mice, or rats (Dumelin et al., 1978a; Dumelin et al., 1978b; Sagai et al., 1981; Sagai et al., 1982). Ethane and pentane can arise from the iron-catalyzed peroxidative breakdown of respectively ω_3 and ω_6 unsaturated fatty acids (Figure 4) (Logani and Davies, 1980; Tappel and Dillard, 1981).

However, up to now, it is not known whether the initial reaction, leading to the possible oxidative breakdown of cellular membrane lipids, is the same for both ozone or nitrogen dioxide - i.e. an allylic hydrogen abstraction -, or that ozone mediated lipid oxidation is initiated by the ionair Criegee mechanism, leading to ozonide intermediates which may possibly only in second instance give rise to radical mediated lipid peroxidation.

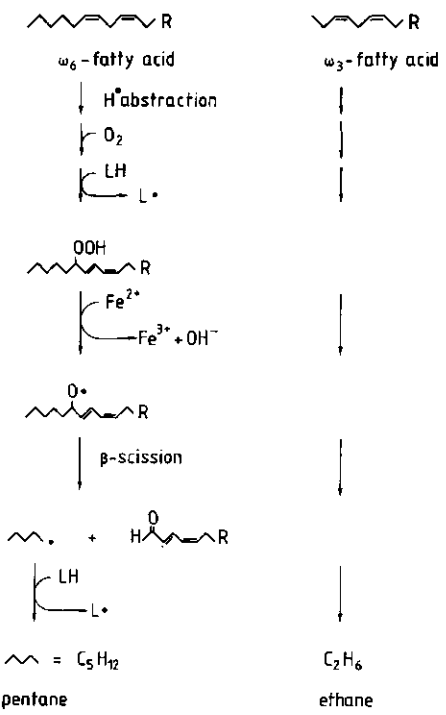

Figure 4. Formation of volatile hydrocarbons (ethane and pentane), following peroxidation of respectively ω_3 and ω_6 unsaturated fatty acids.

In addition to the cellular disruption possibly caused by lipid oxidation, further cellular damage may be induced by the toxic products arising from this oxidation. Lipid hydroperoxides as well as lipid ozonides are known to be very toxic (Cortesi and Privett, 1972; Menzel et al., 1975a; Menzel et al., 1975b; Calabrese et al., 1982; Calabrese et al., 1983), but other products arising from lipid peroxidation may also represent a mechanism by which deleterious effects on cellular functions can be caused.

Frankel et al. (1977a; 1977b) demonstrated that in addition to the lipid hydroperoxides minor products such as fatty acid epoxides, alcohols, aldehydes and ketones arise from autoxidation as well. And

Benedetti et al. (1984a; 1984b) described a group of toxic aldehydes, 4-hydroxy alkenals (R-CHOH-CH=CH-CHO), arising from peroxidation of liver microsomal lipids and able to react with SH-groups of low molecular weight thiols and proteins.

2.2. Oxidation of proteins

In addition to the ozone or nitrogen dioxide induced oxidation of the unsaturated fatty acid moieties of membrane lipids, oxidation of cellular proteins possibly contributes to the induced cell damage as well. Oxidation of sensitive amino acids of enzymes may lead to inhibition of their catalytic function (Chio and Tappel, 1969; Mudd et al., 1969; Buckley et al., 1975; Mudd and Freeman, 1977; Freeman et al., 1979), and oxidation of structural membrane proteins may result in a loss of membrane function.

As with the theories for oxidation of the unsaturated fatty acid moieties in membrane phospholipids, theories for the involvement of protein oxidation in the toxic mechanism of action of ozone or nitrogen dioxide are primarily based on *in vitro* studies under a-biotic conditions. From *in vitro* studies with amino acid solutions it appeared that several amino acids were oxidized by ozone or nitrogen dioxide. Their relative sensitivity towards oxidation varied in the order cysteine > methionine > tryptophan > tyrosine > histidine > cystine > phenylalanine. Other amino acids were unaffected (Mudd et al., 1969).

Obviously this order of reactivity may be somewhat different for amino acid residues of proteins, where the position of a susceptible amino acid residue in the tertiary structure of the protein can affect its sensitivity.

The amino acid residues in proteins may be damaged either by direct oxidation or by a mechanism initiated by hydrogen abstraction (Stokinger, 1965; Menzel, 1971; Hornsby and Crivello, 1983; Hoey and Butler, 1984).

The last process may be followed by oxygenation and/or polymerization, analogue to the mechanism for oxidation of unsaturated fatty acids (Figure 1). In theory, this radical mediated protein oxidation could be initiated by nitrogen dioxide or ozone themselves, but it might as well be a process initiated in second instance by lipid radicals such as LOO$^{\bullet}$ (Hoey and Butler, 1984) (Figure 5), or by the reactive intermediates possibly formed upon the reaction of ozone or nitrogen dioxide with water ($O_2^{-\bullet}$: HOO$^{\bullet}$: H_2O_2 ; $^{\bullet}$OH : $^1O_2$) (section 2.1).

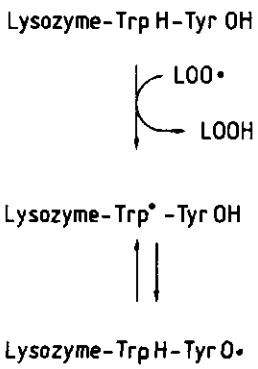

Figure 5. Oxidation of amino acid residues in lysozyme by lipid peroxyl free radicals as suggested by Hoey and Butler (1984).

Radical mediated termination reactions or non-radical mediated oxidations may lead to cross linking of membrane proteins by for example disulfide bridges (De Lucia et al., 1975) or o-o'-dityrosine bridges (Verwey et al., 1982) (Figure 6).

Cross linking of amino acids or proteins has also been considered to arise from a reaction of the proteins with aldehyde products, - such as malondialdehyde - formed during peroxidation of membrane lipids (Chan et al., 1977; Buege and Aust, 1978; Logani and Davies, 1980) (Figure 6 + 7). Some investigators, however, have concluded that the amount of malondialdehyde produced during ozone exposure may be very

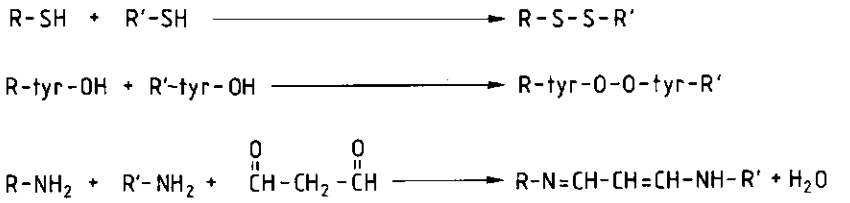

Figure 6. Cross linking of amino acids and/or proteins by disulfide bridges, o-o'-dityrosine bridges or malondialdehyde.

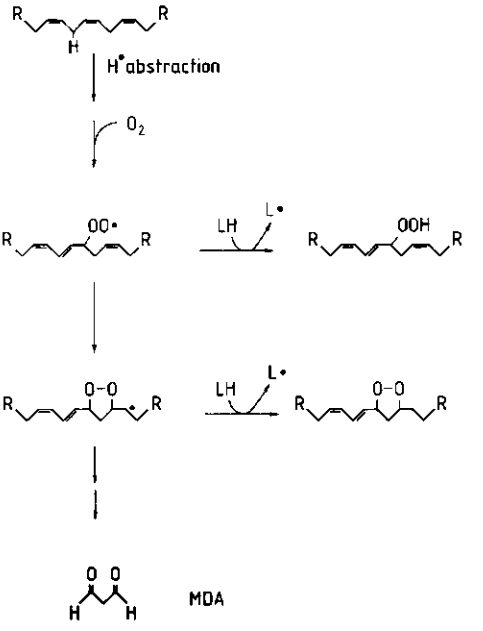

Figure 7. Formation of malondialdehyde (MDA) following peroxidation of polyunsaturated fatty acids.

low and therefore insufficient to bring about protein cross linking (Teige et al., 1974; Freeman et al., 1979).

Evidently cross linking of membrane proteins may have a pronounced impact on both structural and functional properties of the cell membrane.

But, as for the oxidation of unsaturated membrane fatty acids, direct evidence for the involvement of protein oxidation in the toxic mechanism of action of ozone and nitrogen dioxide is lacking. The theories presented are mainly based on in vitro exposures of amino acid or protein solutions.

2.3. Antioxidant protection against ozone and nitrogen dioxide

Several cellular mechanisms of defense have been shown to inhibit the toxic action of oxidants like ozone and nitrogen dioxide. As the toxicity of both gases can be ascribed to their oxidative nature, biological antioxidant systems can be expected to protect both cells in vitro as well as animals or man exposed in vivo, from damage by ozone or nitrogen dioxide.

Protection by the membrane bound antioxidant vitamin E for example has been well documented. Alink et al. (1982) demonstrated the protective action of vitamin E on lung cells exposed to ozone in vitro. And in vivo an increased sensitivity of rat pulmonary tissue after depletion of dietary vitamin E as well as a decreased sensitivity of rats and mice fed a vitamin E supplemented diet, have been demonstrated (Roehm et al., 1971a/b; Fletcher and Tappel, 1973; Donovan et al., 1977; Chow et al., 1979; Menzel, 1979; Chow et al., 1981).

In addition, in vivo evidence for the involvement of the water soluble antioxidant vitamin C (ascorbic acid) in the cellular protection against oxidative air pollutants, is provided by studies demonstrating decreased levels of ascorbic acid in the lungs of exposed animals (Mustafa and Tierney, 1978; Kratzing and Willis, 1980), and by studies in which administration of ascorbic acid to

animals lessened the pulmonary damage caused by subsequent exposure (Matzen, 1957; Pagnotto and Epstein, 1969).

Evidence for the protection against ozone or nitrogen dioxide by the water soluble antioxidant glutathione is indirect, as it is provided by **i)** studies reporting a decrease in reduced glutathione in the lungs or blood of exposed animals (Mountain, 1963; Goldstein et al., 1968a; De Lucia et al., 1972) or man (Buckley et al., 1975), or by **ii)** the observation that exposure of animals for several days to 0.1-1.0 ppm ozone or 2.3-10 ppm nitrogen dioxide enhances the activity of the enzymes of the socalled glutathione peroxidase pathway in their lungs (Chow and Tappel, 1973; Chow et al., 1974; Sagai et al., 1982).

The role of other possible antioxidant systems in the cellular defense against ozone or nitrogen dioxide has not been clearly demonstrated up to now, neither *in vivo* nor *in vitro*.

In the next section, reaction mechanisms available for cellular defense against ozone or nitrogen dioxide are discussed in more detail.

2.4. Mechanisms of cellular defense

The mechanisms available for cellular defense against ozone or nitrogen dioxide may be divided in two main classes, in part analogous to the classification made by Burton and Ingold (1983) and Hornsby and Crivello (1983) based on the role of the antioxidant systems in preventing radical mediated lipid peroxidation.

The first group consists of socalled "**preventive antioxidant systems**", they prevent the formation of initial damage to cellular proteins and/or membrane lipids. The second group contains antioxidant systems which prevent the onset of further cell damage induced by the initially oxidized cell components and might be called "**suppressive antioxidant systems.**"

Antioxidant systems of the first group may act by scavenging the reactive initiating species, or by protecting the target molecules from oxidative attack.

1) An example of this can be found in the structural characteristics of the cell membrane whose hydrophobic phospholipid bilayer may not easily be penetrated by polar oxidative initiating species (Gutteridge, 1978; Dormandy, 1978; Pryor et al., 1983; Hornsby and Crivello, 1983).

2) Similarly, a decreased accessibility of susceptible amino acids, determined by either the cellular location of the protein itself or by the position of the susceptible residue in the tertiary structure of the protein, may contribute to the protection of structural or functional proteins (Pryor et al., 1983).

3) In addition, the formation of socalled mixed disulfides between glutathione and protein sulfhydryl groups might protect these groups from irreversible oxidation by ozone, nitrogen dioxide or their reactive initial intermediates (De Lucia et al., 1972; De Lucia et al., 1975; Chan et al., 1977). Such reactive intermediates might be $O_2^{-\cdot}$, its protonated form $HOO^{\cdot}$, H_2O_2, or $^{\cdot}OH$ free radicals, formed upon the reaction of ozone or nitrogen dioxide with water (section 2.1).

4) Therefore, superoxide dismutase (SOD), catalase and the Se-dependent glutathione peroxidase, which dismutate $O_2^{-\cdot}$ or H_2O_2 (Figure 8) belong to this category of antioxidant systems as well.

5) Finally vitamin E (α-tocopherol), vitamin C (ascorbic acid) and glutathione also belong to this group of preventive antioxidant systems, as far as their capacity to scavenge the reactive initial compound is concerned.

Suppressive antioxidant systems on the other hand, prevent the onset of further cellular damage by the initially oxidized cell components. Such antioxidant systems may act by converting these oxidized cell components to their initial reduction state or to less toxic intermediates.

- Superoxide dismutase (SOD)
  - *Mn-SOD : mitochondria
  - *Cu/Zn-SOD : cytoplasm

$$SOD\text{-}Cu^{2+} + O_2^{\cdot} \longrightarrow SOD\text{-}Cu^{+} + O_2$$
$$SOD\text{-}Cu^{+} + O_2^{\cdot} + 2H^{+} \longrightarrow SOD\text{-}Cu^{2+} + H_2O_2$$
$$O_2^{\cdot} + O_2^{\cdot} + 2H^{+} \longrightarrow O_2 + H_2O_2$$

- Catalase

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

- Se-dependent GSH-peroxidase

$$H_2O_2 + 2GSH \longrightarrow 2H_2O + GSSG$$

Figure 8. Detoxification of superoxide anion free radicals ($O_2^{\cdot}$) by superoxide dismutase and of hydrogen peroxide (H_2O_2) by catalase or Se-dependent glutathione peroxidase.

1) The glutathione peroxidase enzyme system can act in this way, reducing the toxic lipid hydroperoxides to their corresponding alcohols using the reduction equivalents of glutathione (Figure 9). The oxidized glutathione formed in this way, can be reduced by glutathione reductase using NADPH reduction equivalents, provided by the hexose-monophosphate shunt.

2) In addition to the glutathione peroxidase system, detoxifying lipid hydroperoxides, enzymes capable of metabolizing other toxic products of lipid peroxidation such as epoxides, aldehydes or ketones might as well prevent the onset of further cellular damage. Among these are the enzymes of xenobiotic metabolism such as epoxide hydrolase (Mead, 1980; Hornsby and Crivello, 1983), aldehyde dehydrogenase (Slater, 1979; Benedetti et al., 1984b) and maybe mono-oxygenases and conjugating enzymes as well (Slater, 1979; Hornsby and Crivello, 1983).

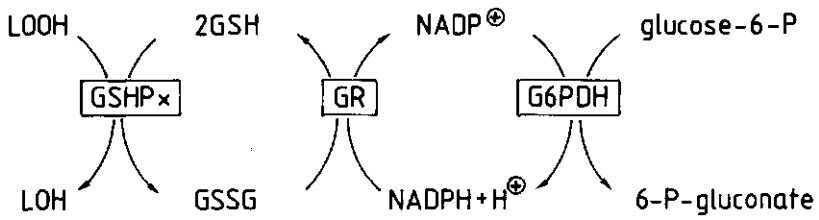

Figure 9. Detoxification of lipid hydroperoxides (LOOH) by the glutathione peroxidase pathway.
GSHPx = glutathione peroxidase, GR = glutathione reductase and G6PDH = glucose-6-phosphate dehydrogenase.

3) Metal chelating proteins such as ferritin, transferrin and lactoferrin, are part of this group of antioxidant systems as well because they may prevent the metal-catalyzed homolytic cleavage of (lipid) hydroperoxides to toxic free radical species (Figure 1) by binding the catalytic transition metal ions (Buege and Aust, 1978; Winterbourn, 1981; Gutteridge et al., 1981; Hornsby and Crivello, 1983). In addition, enzymes such as caeruloplasmin may prevent this cleavage by keeping the metal ions in their oxidized (i.e. catalytically less active) state (Hornsby and Crivello, 1983; Brinkman et al., 1964; Dormandy, 1978; Cranfield et al., 1979).

4) Finally antioxidants like α-tocopherol, vitamin C and glutathione may stop the process of radical mediated lipid peroxidation by scavenging the propagating radicals LOO$^{\cdot}$ and L$^{\cdot}$.

On the following pages the cellular antioxidant systems on which attention was focussed in this thesis are discussed in more detail.

VITAMIN E

Emerson et al. (1937) demonstrated that vitamin E is made up of a group of closely related tocopherols, named α,β , γ and δ -tocopherol (Figure 10). The first one, α -tocopherol turned out to be the one

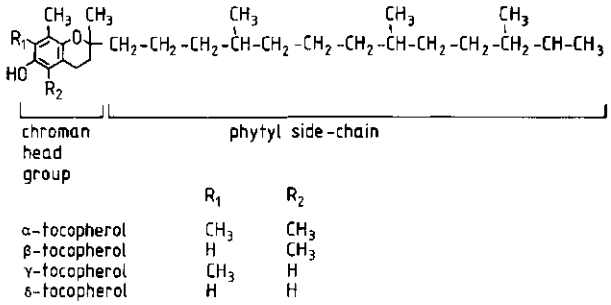

Figure 10. Molecular structure of tocopherols.

most common and also the most active member of this group with respect to both its biological and its in vivo antioxidant activity (McCay and King, 1980; Burton and Ingold, 1983; McCay, 1985). The antioxidant action of this membrane bound cell component is generally ascribed to its ability to transfer a hydrogen free radical to a lipid (peroxyl) free radical:

$$L^{\bullet} + AOH \longrightarrow LH + AO^{\bullet}$$
$$LOO^{\bullet} + AOH \longrightarrow LOOH + AO^{\bullet}$$

thus terminating the peroxidative chain reaction (Burton and Ingold, 1983; McCay, 1985).

Obviously other free radicals can be scavenged as well. Vitamin E was shown to scavenge $O_2^{\bullet-}$ radicals (Ozawa et al., 1978; Nanni et al., 1980; Tajima et al., 1983; Fukuzawa and Gebicki, 1983), its protonated from $^{\bullet}OOH$ (Fukuzawa and Gebicki, 1983), $^{\bullet}OH$ radicals (Fukuzawa and Gebicki, 1983) and even tryptophan free radicals (Hoey and Butler, 1984).

The most reactive hydrogen atom of vitamin E was shown to be that of its hydroxyl group (Boguth and Niemann, 1971; Urano and Matsuo, 1976; Ozawa et al., 1978). The chromanoxyl free radical formed upon hydrogen donation by α-tocopherol, appears to be relatively stable and unreac-

tive due to resonance stabilization (Boguth and Niemann, 1971; Urano and Matsuo, 1976; Ozawa et al., 1978).

As vitamin E is a two electron donor, the chromanoxyl free radical of α-tocopherol might donate a second hydrogen atom, scavenging a second radical. This second hydrogen donation by the chromanoxyl free radical of α-tocopherol may proceed as depicted in Figure 11. The reaction may give rise to the generation of α-tocopherol quinone (Shimasaki and Privett, 1975), or of the other oxidized form depicted in Figure 11 (Pryor, 1976). However, when the chromanoxyl free radical is transformed into a benzyl free radical by a socalled chromanoxyl-benzyl-radical-rearrangement (Boguth and Niemann, 1971) the second hydrogen free radical may also be provided by the hydroxyl group of the chroman head group (Figure 11).

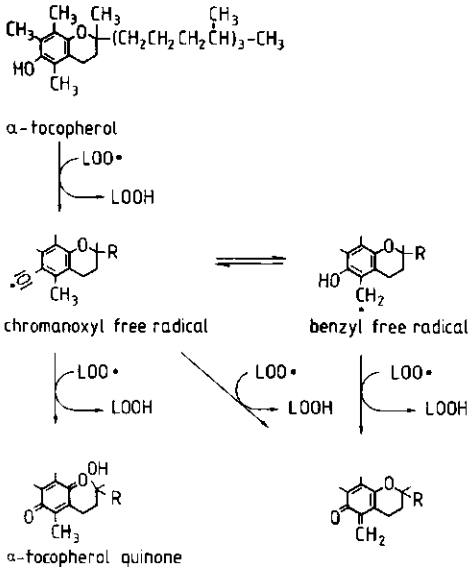

Figure 11. Hydrogen donation reactions / one electron oxidation reactions, of α-tocopherol.

Instead of donating a second hydrogen atom, the α-tocopherol chromanoxyl free radical may be repaired by another cellular hydrogen donor, such as vitamin C or glutathione, a process described in section 2.5 and resulting in regeneration of the α-tocopherol molecule.

Besides this radical scavenging capacity, α-tocopherol may also act as an antioxidant by quenching toxic non-radical species such as for example the reactive singlet oxygen molecule (Grams and Eskins, 1972; Foote et al., 1978; McCay et al., 1978; Chow et al., 1979).
Quenching of singlet oxygen and the radical scavenging action of α-tocopherol can also produce oxidation products different from the ones described above. Quenching of singlet oxygen by α-tocopherol was reported to yield a complex mixture of oxidized quinones and quinone-epoxides (Grams and Eskins, 1972; Foote et al., 1978), whereas the oxidation products of α-tocopherol detected in the presence of autoxidizing lipids also include a dimer and even a trimer of α-tocopherol as well as adducts of the oxidized tocopherol with fatty acid moieties of the lipids (Matsushita et al., 1978).

In addition to its action as an antioxidant, Lucy and Dingle (1964) have proposed another mechanism that may contribute to the protective role of vitamin E, namely its action as a membrane stabilizer. Based on molecular building studies, it was suggested that vitamin E might stabilize membrane structures by virtue of a specific physico-chemical interaction between its phytyl side-chain and the fatty acyl chains of polyunsaturated phospholipids, particularly those with arachidonyl fatty acid residues (Lucy and Dingle, 1964; Lucy, 1972; Diplock and Lucy, 1973) (Figure 12). Such a close physico-chemical association may provide a structural mechanism for protection of these polyunsaturated membrane phospholipids against peroxidative breakdown (Lucy and Dingle, 1964; Lucy, 1972; Diplock and Lucy, 1973).

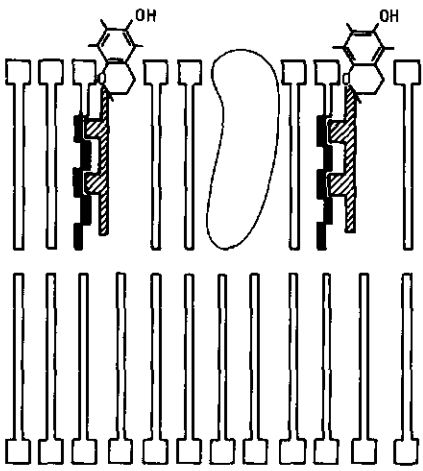

Figure 12. Schematic representation of the structural membrane stabilization by α-tocopherol provided by the physico-chemical interaction between its phytyl side chain and the fatty acyl chains of polyunsaturated membrane phospholipids, as suggested by Lucy (1972) and Diplock and Lucy (1973).

VITAMIN C

The second cellular antioxidant of importance, vitamin C (ascorbic acid), is water soluble and present in the cytoplasm of the cell. Like vitamin E, vitamin C is a two electron / hydrogen donor.

In biological systems, at neutral pH, ascorbic acid exists in its dissociated form (Swartz and Dodd, 1981) (Figure 13).

The ascorbate anion may act as an electron donor in a radical scavenging reaction, giving rise to the ascorbyl free radical, also called mono-dehydroascorbic acid (Bielski et al., 1975; Swartz and Dodd, 1981) (Figure 13). At physiological pH, the ascorbyl free radical is mainly present in its dissociated, anionic form and appears to be relatively inert because of its resonance stabilization (Laroff et al., 1972; Bielski et al., 1975; Swartz and Dodd, 1981) (Figure 13). Nevertheless the ascorbyl free radical may quench a second radical giving rise to dehydroascorbic acid (Figure 13) or it may

react with another ascorbyl free radical, disproportionating into ascorbic acid and dehydroascorbic acid (Bielski et al., 1975; Swartz and Dodd, 1981).

Finally regeneration to ascorbic acid may occur, either enzymatically at the cost of NADH reduction equivalents catalyzed by the NADH-semidehydroascorbate reductase system (Ito et al., 1981) or chemically by other cellular electron / hydrogen donors (section 2.5).

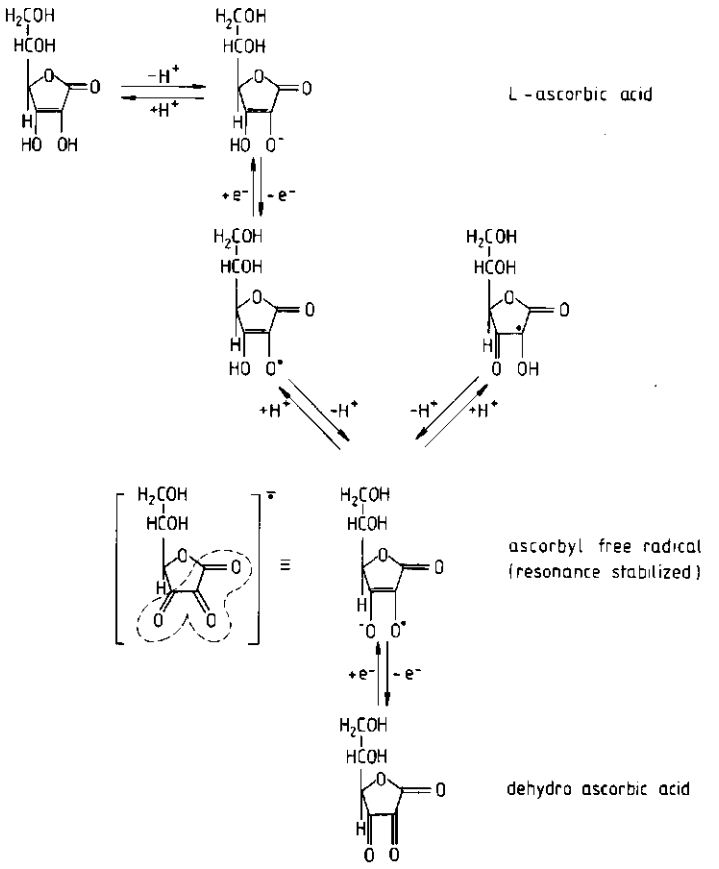

Figure 13. One electron oxidation reactions of ascorbic acid.

Like vitamine E, vitamin C may scavenge radicals from the lipid-peroxidative chain reaction, thus preventing formation of increased amounts of lipid hydroperoxides, but vitamin C is also capable of quenching other radicals such as $O_2^{\cdot-}$ (Nishikimi, 1975; Nanni et al., 1980), $^{\cdot}OH$ (Laroff et al., 1972; Fessenden and Verma, 1978), and even some amino acid free radicals, viz. tryptophan or tyrosine derived free radicals either in their free form or even when incorporated into a protein molecule.

The capacity of the polar ascorbic acid molecule to quench lipid peroxyl free radicals present in the apolar cell membrane, might be declared by the point stated by Barclay and Ingold (1980) that lipid peroxyl free radicals, having a significant dipole moment might move out of the inner membrane region (Hornsby and Crivello, 1983).

GLUTATHIONE

As vitamin C, the tripeptide glutathione (γ-Glu-CysSH-Gly) (GSH) is a water soluble antioxidant present in the cytoplasm of cells. Its antioxidant capacity could be due to a direct antioxidant function, comparable to the one described for vitamin E and C, but it might as well be ascribed to its substrate function in a number of enzyme catalyzed cellular reactions, like the detoxification of peroxides.
The most important enzyme involved may be glutathione peroxidase (GSHPx) which catalyzes the reduction of peroxides to the corresponding alcohols using glutathione reduction equivalents (Chow and Tappel, 1972) (Figure 9). Two different forms of GSHPx have been demonstrated to occur, a selenium(Se)-dependent GSHPx and a Se-independent one, which might be related to GSH S-transferase (Flohé, 1982).
The main difference between both forms of GSHPx can be found in the fact that only the Se-dependent GSHPx is able to reduce H_2O_2. Both GSHPx-es are able to reduce organic hydroperoxides although with a different substrate affinity, Km being 1-10 μM for the Se-dependent

GSHPx and 0.5-2.5 mM for the Se-independent form (Little et al., 1970; Lawrence et al., 1978).

The ability of GSHPx to reduce toxic fatty acid hydroperoxides incorporated in membrane phospholipids, has been doubted by McCay et al. (1976), who reported that the GSHPx system was not able to reduce the lipid hydroperoxides present in phospholipid emulsions to the corresponding alcohols. In a biological system, however, the action of GSHPx on hydroperoxide moieties in membrane phospholipids may depend on the action of phospholipases which may recognize peroxidized membrane associated lipid chains and cleave them, resulting in their release into the cytoplasm where they may be reduced by GSHPx (Grossmann and Wendel, 1983; Flohé, 1982).

Besides its substrate function in GSHPx-catalyzed reactions, glutathione could possibly be used for the formation of socalled "mixed disulfides".

This reaction can be catalyzed by GSH transferases and results in the formation of disulfide linkage between glutathione and a protein thiol group. In this way important thiol groups of structural and/or functional proteins might be reversibly protected from irreversible oxidation by oxidative compounds (De Lucia et al., 1972; De Lucia et al., 1975).

Furthermore, several less well defined glutathione dependent enzyme systems capable of protecting membranes, have been described. Among them are cytosolic as well as membrane associated and microsomal protein factors (McCay et al, 1981; Ursini et al., 1982; Burk, 1983; Hill and Burk, 1984).

In addition to these glutathione dependent enzyme catalyzed antioxidant systems, glutathione may function as a direct scavenger of reactive intermediates as for example $^{\cdot}OH$, $O_2^{\cdot-}$ or carbon centered free radicals (Forni and Willson, 1983). The thiyl radical of glutathione ($GS^{\cdot}$), formed upon a radical scavenging reaction is relatively stable and causes no further damage (Forni and Willson, 1983). It might recombine with another $GS^{\cdot}$ to give GSSG (Pryor, 1976), but in a

biological system it might also react with a reducing agent (for example vitamin C, NAD(P)H or cytochrome C) in an electron transfer reaction giving rise to the thiol anion GS^-, which - in aqueous surroundings at physiological pH - immediately protonates to give GSH (Forni and Willson, 1983) (Figure 14).

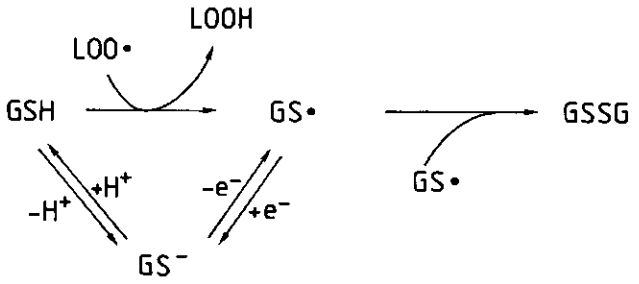

Figure 14. Chemical oxidation and reduction reactions of glutathione as suggested by Pryor (1976) and Forni and Willson (1983).

2.5. Interactions between different cellular antioxidant systems

During oxidative stress all cellular antioxidant systems available might cooperate in protecting the cell from irreversible oxidative damage. There is evidence, for example, that lipid peroxyl free radicals can be scavenged by α-tocopherol (Menzel, 1970; Packer et al., 1979; Burton and Inghold, 1983; McCay, 1985), and that the lipid hydroperoxides formed, can be detoxified by glutathione peroxidase after they have been liberated from the cell membrane by phospholipases, (Grossman and Wendel, 1983). The α-tocopherol free radical formed might be reduced by other antioxidants, for example vitamin C (Menzel, 1970; Packer et al., 1979; Leung et al., 1981; McCay, 1985)., which - in turn - may be regenerated by a NADH dependent reductase (Packer et al., 1979; Ito et al., 1981)., or - after its dispropor-

tionation - by glutathione (Menzel, 1970). Finally, the oxidized glutathione formed in this reaction can be regenerated by glutathione reductase using NADPH reduction equivalents. All these processes are depicted in Figure 15.

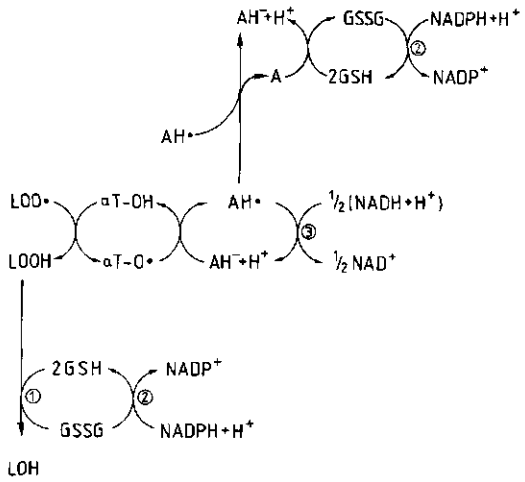

Figure 15. Example of a sequence of cellular redox couples demonstrating the possible cooperation between several antioxidant systems.
AH = ascorbic acid, αT-OH = α-tocopherol.
1 = GSH peroxidase, 2 = GSSG reductase, 3 = semidehydro ascorbic acid reductase.

This sequence of redox-couples is the one usually put forward, because the redox-couples involved have been demonstrated to occur in vitro either enzyme catalyzed or non-catalyzed (Menzel, 1970; Mustafa and Tierney, 1978; Packer et al., 1979; Ito et al., 1981). But in addition to the sequences mentioned in Figure 15 other redox-couples may occur. Vitamin C, for example, or glutathione, may scavenge lipid peroxyl free radicals as well (Menzel, 1970; Hornsby and Crivello, 1983; McCay, 1985) and the α -tocopherol free radical may perhaps be regenerated directly by glutathione, without the intervention of vitamin C (Pryor, 1976).

Obviously it may even be possible that all redox couples of cellular electron / hydrogen donors cooperate, keeping all of them in balance as good as possible. Thus it might happen that the vitamin C free radicals are regenerated at the cost of glutathione reduction equivalents, but - when glutathione is primarily acting as the electron / hydrogen donor -, that the reaction predominantly proceeds in the opposite direction, resulting in regeneration of the glutathione thiyl free radical by vitamin C (Forni and Willson, 1983). In addition, the thiyl free radical of glutathione might also be reduced by other cellular electron / hydrogen donors such as NADH, or cytochrome C (Forni and Willson, 1983).

Equilibria between all cellular electron / hydrogen donors will be determined by their respective redoxpotentials, their cellular concentrations, the overall result of their enzyme catalyzed or chemical conversions, but also by structural characteristics as for example the ability of different electron / hydrogen donors to reach each other within the cell.

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CHAPTER 3

IN VITRO MODEL SYSTEMS FOR STUDYING THE TOXICITY OF GASEOUS COMPOUNDS

The selection of a model system for in vitro studies includes the choice of i) an appropriate exposure model and ii) appropriate cell types that can be used.
In this chapter these two aspects of in vitro toxicity studies are discussed in more detail and the in vitro system used throughout this thesis is described.

3.1. Description of the methods available for in vitro exposure to gaseous compounds

Several in vitro model systems for exposure of cells to gaseous compounds have been described in the literature. Generally they can be divided into four categories, depending on the way in which the gaseous compound and the culture medium reach the cells. These categories include 1) dry air exposure models, 2) liquid mediated exposure models, 3) liquid diffusion mediated exposure models and 4) gas diffusion mediated exposure models. Figure 1 gives a schematic representation of all four categories, who are discussed in more detail on the next pages.

Dry air exposure models

Dry air exposure models include all systems in which cells are alternatively exposed to the gaseous atmosphere or the culture medium. This can be achieved by culturing a monolayer of cells in a culture flask or dish and exposing them alternatively to the culture medium or the gaseous atmosphere by inverting or rotating the flask or by turning the culture dishes (Figure 1). The influx of gas into the system can be either i) once, e.g. only before exposure, closing the flask or the chamber into which the culture dishes are placed after

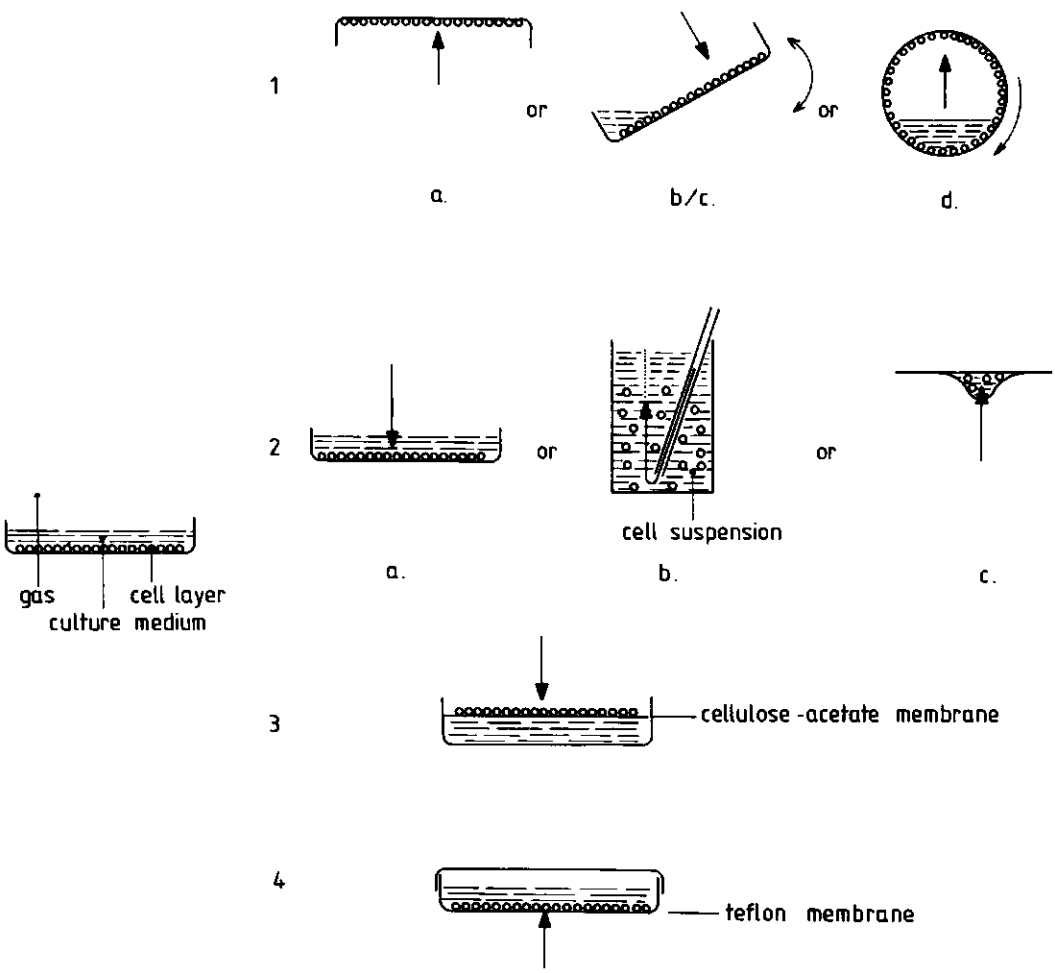

Figure 1. Schematic representation of:

1) dry air exposure models
   a) inverted culture flasks
   b) dishes on rotating platforms
   c) dishes on rocker platforms
   d) roller bottles
2) liquid mediated exposure models
   a) cell cultures overlaid with medium
   b) gas bubbling
   c) hanging drop
3) liquid diffusion mediated exposure models
4) gas diffusion mediated exposure models

⟶ represents O_3/NO_2

addition of the gas, or ii) continuously, using specially designed roller caps for culture flasks or specially designed exposure chambers.

Examples of dry air exposure systems described in the literature are culture flasks which are alternatively inverted (Pace et al., 1961; Pace et al., 1969), roller bottle cultures (Baker and Tumasonis, 1971; Bolton et al., 1982) and culture dishes on rocker platforms (Rasmussen, 1986) or on rotating platforms (Valentine, 1985).

An advantage of these systems is that the ozone and nitrogen dioxide concentrations applied can be expressed in ppm's and thus compared to concentrations known to induce toxic effects in vivo. Reports in the literature have described toxic effects of ozone and nitrogen dioxide in these dry air exposure models at concentrations of 0.2 - 6 ppm ozone or 5 ppm nitrogen dioxide which is in the same order of magnitude as the concentrations known to cause toxic effects in vivo (Pace et al., 1961; Pace et al., 1969; Valentine, 1985; Rasmussen, 1986).

A disadvantage of these systems is that the impact of drying of the cells is difficult to control, resulting in a risk for drying of the cells. Another drawback is that the continuous movement of medium over the cell layer may dislodge some cells, particularly the damaged ones, a phenomenon that may influence the results.

Liquid mediated exposure models

The liquid mediated exposure of cell cultures to gaseous compounds relies on the solution of the gases into the medium overlaying or surrounding the cells. Examples of liquid mediated exposure models are the exposure of cell cultures overlaid with medium, the model in which a cell suspension is exposed by means of gas bubbling and the model in which a cell suspension is exposed to a gaseous atmosphere as a drop hanging in a specially designed exposure chamber (Pace et al., 1961; Goldstein and Balchum, 1967; Pace et al., 1969; Weissbecker et al., 1969; Goldstein et al., 1977; Sweet et al., 1980; Hagar et al., 1981;

Sone et al., 1983; Konings, 1986).

The advantage of some of these systems is that cells are exposed under optimal conditions for cell growth. This advantage does not completely hold for the exposure by means of gas bubbling as there is a risk for mechanical damage to the cells during exposure in this model system. A disadvantage of all these systems is that they do not seem to be representative for the in vivo situation because there is a thick liquid phase between the cells and the gaseous atmosphere. In addition, it is almost impossible to assess the concentration of the oxidative gases in the medium near the cell surface and therefore concentrations used to specify exposure conditions are the concentrations present in the gaseous atmosphere from which the gases dissolve into the liquid phase. In general relatively high concentrations of the oxidative gases are necessary to induce cytotoxic effects because of i) the relatively low solubility of the oxidative gaseous compounds into the liquid phase and because of ii) the reaction of the gas with medium components. Concentrations reported to induce cytotoxic effects in these liquid mediated model systems amount to 10-25 ppm ozone or 200-8600 ppm nitrogen dioxide (Pace et al., 1961; Weissbecker et al., 1969; Konings, 1986).

For all these reasons we agree with Rasmussen (1984) who concluded that "systems that rely on solution of the gases in the overlaying medium . . . . . may not be suitable for studying effects of the poorly soluble oxidant gases".

Liquid diffusion mediated exposure models

In these model systems cells are cultured on special cellulose acetate filters. The cells are directly and continuously exposed to the gaseous atmosphere and nourished and protected from drying during the exposure by diffusion or perfusion of the culture medium through the filter (Figure 1) (Voisin et al., 1977; Samuelsen et al., 1978; Rasmussen and Crocker, 1981).

An advantage of these systems is that - in analogy to the dry air exposure models - ozone and nitrogen dioxide concentrations to which the cells are exposed can be expressed in ppm's and be compared to concentrations known to induce toxic effects in vivo.
In general cytotoxic effects in these exposure systems have been described at concentrations amounting to 0.05 - 3.3 ppm ozone or to 0.12 - 1 ppm nitrogen dioxide (Samuelsen et al., 1978; Voisin et al., 1981; Rasmussen and Crocker, 1981). From these data it is evident that cells exposed in these liquid diffusion mediated models are sensitive to extremely low concentrations of the oxidative gaseous compounds. This suggests that cells grown on cellulose acetate membranes may not be under optimal culture conditions. This is strengthened by the observation that control cells grown on cellulose acetate membranes survive for only 3 to 4 days (Voisin et al., 1981) and by the fact that Samuelsen et al. (1978) mentioned a small but evident loss of colony forming ability of cells grown on cellulose acetate filters after exposure for 8 hours to control i.e. clean air, conditions. These probably sub-optimal conditions for cell growth for cells grown and exposed on cellulose acetate filters can be a drawback when the system has to be used for mechanistic studies as described in this thesis.

Gas diffusion mediated exposure models

In the gas diffusion mediated exposure model described by Alink et al. (1979) and used for the experiments described in this thesis, exposure is achieved by means of gas diffusion through a gas permeable plastic film on which the cells are grown (Figure 1).
Advantages of this system are that cells are kept under normal culture conditions during the exposure, and that the morphology of the cells can be studied light microscopically.
Another argument in favour of this in vitro exposure model can be found in the fact that ozone appeared to be about 10 times more toxic than nitrogen dioxide in this system (Rietjens et al., 1986, chapter 4,

this thesis). This is a difference comparable with the one reported for *in vivo* studies in which ozone is demonstrated to be about 10 to 25 times more toxic than nitrogen dioxide. Other *in vitro* studies, however, reported ozone to be more toxic than nitrogen dioxide by a factor ranging from 1 (Voisin et al., 1980; Voisin et al., 1981) to about 3000 (Weissbecker et al., 1969) probably depending on the *in vitro* exposure model used.

For studies on the mechanisms of toxic action and cellular defense - as described in this thesis - the advantages of this system compensate for the drawback that exposure levels can not be expressed in ppm, but have to be given as an amount of ozone or nitrogen dioxide that diffuses through the plastic film into the dish. In the next section however, where the exposure model is described in more detail, an attempt is made to compare exposure conditions in this model system, expressed in nmol of ozone or nitrogen dioxide that diffuses into the culture dishes, to ppm exposure levels for *in vivo* experiments.

Finally, Table 1 summarizes the main characteristics of all exposure models described.

From these data it can be seen that the gas diffusion mediated exposure model developed at our laboratory and used for the experiments described in this thesis, is perfectly suitable for experiments on the mechanisms of toxic action of and cellular defense against ozone and nitrogen dioxide. Especially because the cells can be kept at optimal conditions for cell growth before and during exposure.

3.2. The *in vitro* exposure system used for the experiments in this thesis

Throughout this study cells were exposed to ozone and nitrogen dioxide *in vitro*, using the membrane mediated exposure model described by Alink et al. (1979). Cells were cultured on plastic film dishes (Petriperm, Heraeus, Hanau, West Germany) and exposed in the presence of culture medium or a balanced salt solution, by means of gas diffusion through the thin teflon film.

TABLE 1. MAIN CHARACTERISTICS OF 4 CATEGORIES OF IN VITRO SYSTEMS FOR THE EXPOSURE OF CELLS TO GASEOUS COMPOUNDS

characteristics	dry air	liquid mediated	liquid diffusion mediated	gas diffusion mediated
way in which the gas reaches the cells	* direct contact between cells and gaseous compound	* thick liquid layer between cells and gaseous compound	* direct contact between cells and gaseous compound	* direct contact between cells and gaseous compound
conditions for cell growth during exposure	* sub-optimal * risk for - drying of cells - mechanical damage	* optimal * risk for mechanical damage with gas bubbling	* sub-optimal	* optimal
concentration range inducing cytotoxic effects	* O_3: 0.2-6 ppm * NO_2: 5 ppm	* O_3: 10-25 ppm * NO_2: 200-8600 ppm	* O_3: 0.05-3.3 ppm * NO_2: 0.12-1 ppm	* O_3: 5-25 nmol/dish * NO_2: 50-200 nmol/dish
toxicity O_3: NO_2	* 1 - 25	* 10 - 3000	* 1 - 3	* 8 - 12

Before exposure the film dishes were closed in such a way that no ozone or nitrogen dioxide entered the dish except by diffusion through the teflon film. For gas exposure the dishes were placed in a holder in the centre of a 36 l perspex fumigation box, equipped with an electric fan to achieve an even distribution of the gas mixture (Figure 2).

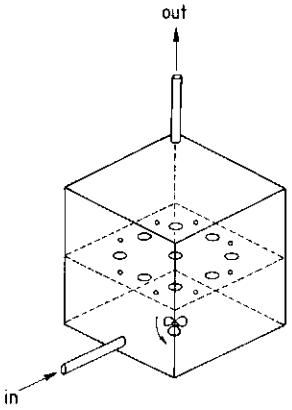

Figure 2. The fumigation box.

During exposure the inlet gas mixture contained 85% N_2: 10% O_2 and 5% CO_2 (all from gas cylinder supplies) (HoekLoos, Schiedam, The Netherlands) and an experimental amount of either ozone or nitrogen dioxide. Ozone was generated from O_2 by a high voltage electric discharge ozone generator (Fisher, Model 501, Meckenheim, West Germany). Nitrogen dioxide was from a cylinder containing 0.9% nitrogen dioxide in nitrogen (Matheson, Oevel, Belgium). The flow of all gas streams could be adjusted separately. The total flow of the gas mixture entering the fumigation box was 2.1 liter/minute for ozone exposures and 1.0 liter/minute for exposures to nitrogen dioxide. Figure 3 shows a schematic presentation of the experimental set up.

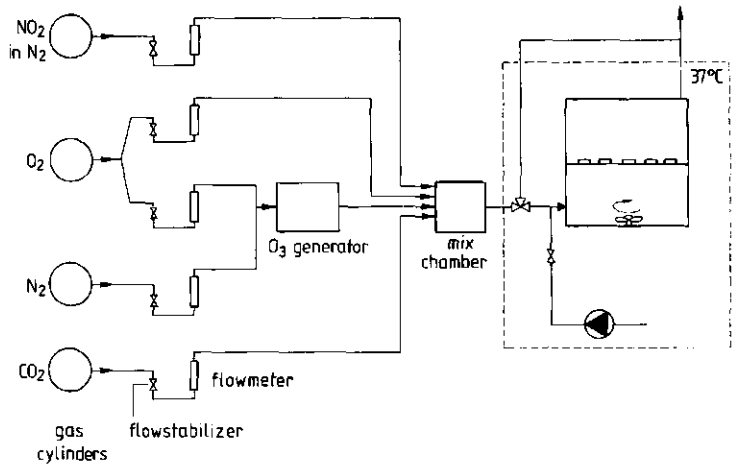

Figure 3. Exposure scheme.

Based on the flow rate of the gas mixture entering the fumigation box and the volume of this box, concentration profiles during the exposures can be calculated from the equation:

$$C_t = (1 - e^{-t/\tau}) . C_{in} \qquad 1$$

with: t = time in minutes

C_t = concentration of the gas (ozone or nitrogen dioxide) after t minutes

C_{in} = concentration of ozone or nitrogen dioxide in the inlet gas mixture

τ = time (in minutes) the gas mixture remains in the fumigation box

$$= \frac{\text{volume of the box (liters)}}{\text{flow rate of the gas mixture (liter/min)}}$$

Exposures were usually carried out for two hours. Then clean air was blown through the fumigation box with a rotor pump (Charles Austen pumps LTD., Weybridge, Surrey, England) (flow = 14.7 liter/min), for about 30 minutes, resulting in a concentration decrease that can be described by the equation:

$$C_{t'} = C_t \cdot e^{-t'/\tau'} \qquad 2$$

with: t' = time during which clean air is blown through the box

$C_{t'}$ = the concentration of the gas (ozone or nitrogen dioxide) after t' minutes

C_t = concentration of ozone or nitrogen dioxide at the end of the exposure

τ' = time (in minutes) the clean air mixture remains in the box

$$= \frac{\text{volume of the box (liters)}}{\text{flow rate of the clean air (liter/min)}}$$

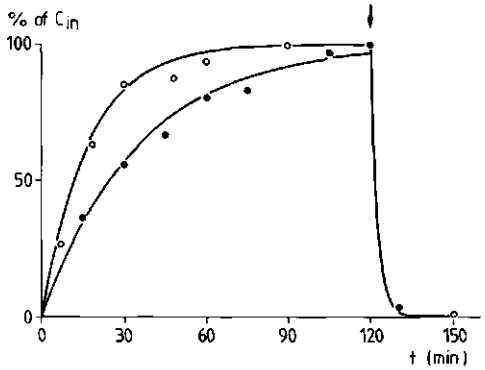

Figure 4. Concentration profiles of ozone (-o-) and nitrogen dioxide (-●-) in the fumigation box, measured in the air leaving the box, and given as percentage of C_{in} which represents the concentration of the oxidative compound in the inlet gas mixture.

The solid curves represent concentration profiles calculated from the equations given in the text, with a flow of 2.1 liter/minute for ozone and of 1.0 liter/minute for nitrogen dioxide exposure.

At (↓) clean air is blown through the fumigation box with a flow of 14.7 liter/minute.

Figure 4 shows the concentration profiles as a function of exposure time for both ozone and nitrogen dioxide exposures as calculated from equations 1 and 2 (solid curves).

Measurement of the oxidant concentrations in the air leaving the fumigation box confirmed these calculated concentration profiles (Figure 4).

The amount of ozone or nitrogen dioxide that can reach the cells on the teflon film of the petriperm dish, is determined by the amount of the gas that diffuses through the teflon film into the dish.

This gas diffusion through a polymeric teflon membrane can be quantitatively described by Fick's law (Stannett, 1968; Karel, 1975):

$$Q = D.\ A.\ \frac{\Delta C}{\Delta X}$$

with: Q = diffusion rate of the gas in mol/sec
A = area through which diffusion takes place in cm^2
D = diffusion coefficient of the gas in the membrane in cm^2/sec
Δc = concentration difference over the membrane in $mol/cm^3/sec$
Δx = thickness of the membrane in cm.

The diffusion coefficient (D) is known to be dependent on the following parameters (Stannett, 1968):

- temperature
- characteristics of the polymer
- characteristics of the gas i.e.
  - molecular diameter of the gas
  - polarity of the gas molecule.

These parameters and thus the diffusion coefficient, as well as the surface area and thickness of the teflon film and the temperature, are the same for all exposures to a specific gaseous compound. Therefore the amount of ozone or nitrogen dioxide that diffuses through the teflon film into the dish and thus the actual amount to which the cells can be exposed, will increase in a linear way with increasing concentrations of the gas in the fumigation box. This was demonstrated to be true for both ozone and nitrogen dioxide exposures in our *in vitro* system (Figure 5).

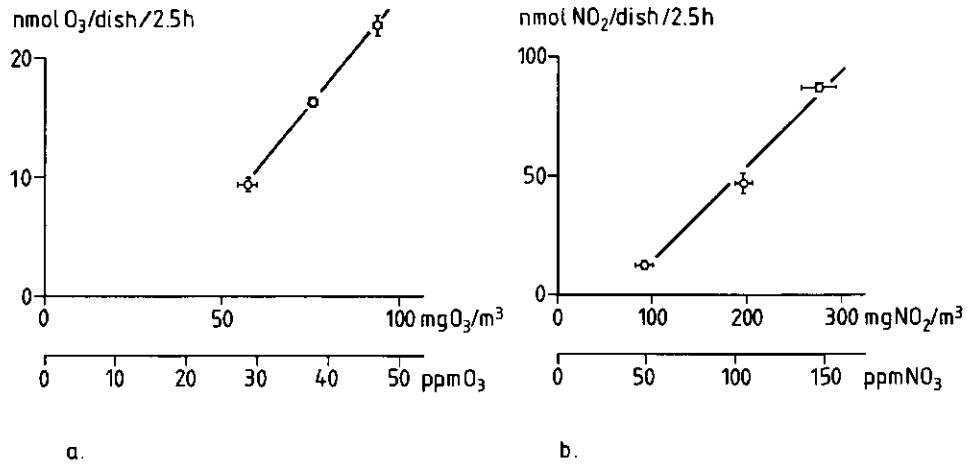

Figure 5. Relation between the concentration of ozone (a) or nitrogen dioxide (b) in the inlet gas mixture and the amount of gas which diffuses into the petriperm dishes.

From the data presented in Figure 5 it can also be concluded that the doses to which cells are exposed in this *in vitro* system have to be expressed in nmol or μg O_3 or NO_2/dish, although the concentrations in the fumigation box itself are given in nmol or $\mu g/m^3$ or in ppm, which is the unit usually applied for ozone or nitrogen dioxide

concentrations encountered in the environment. However, the doses applied in our *in vitro* system expressed in μg or nmol/dish may be compared to the amounts of ozone or nitrogen dioxide that reach the pulmonary tissue during realistic *in vivo* exposures.

Using the model for pulmonary deposition of ozone, presented by Miller et al. (1978) this pulmonary deposition at several experimental ozone concentrations can be calculated. The model predicts that at a tracheal ozone concentration of for instance 200–1000 $\mu g/m^3$ (0.1 – 0.5 ppm), pulmonary deposition amounts to (0.2 – 2.0) x 10^{-5} μ g O_3/cm^2/ breath. From this it can be calculated that with 10–20 breaths per minute, the ozone dose to which lung cells may be exposed during 2.5 h would vary between about (0.3 – 6.0) x 10^{-2} μ g O_3/cm^2/2.5 h. For a petriperm dish with a teflon growth area of 18 cm^2, this would result in an ozone dose of about 0.05 – 1.1 μ g O_3/dish/2.5 h (= 1–23 nmol O_3/dish/2.5 h), which is the range applied in our *in vitro* system.

3.3. Cell cultures applied for *in vitro* studies on oxidative gaseous compounds

In vitro studies may have several advantages for studying toxic effects as compared to studies *in vivo*. Of interest for the experiments described in this thesis is that homogeneous cell populations can be studied instead of the cellular very heterogenic lung with its 40 different cell types.

In vitro effects of oxidative gaseous compounds should preferentially be studied using cells originating from lung tissue, unless it is proven that other, non-lung cell types show a similar response.

Cell cultures originating from lung tissue may involve established cell lines, derived from a specific lung cell population, or primary cultures isolated from freshly prepared lung tissue.

Established lung cell lines commonly used for *in vitro* studies are the V79 Chinese hamster lung fibroblast cell line (Samuelsen et al., 1978; Alink et al., 1979; Rasmussen and Crocker, 1981) and the A549 cell line (Alink et al., 1980; Sweet et al., 1980; Alink et al., 1982).

With respect to the primary lung cell cultures, appropriate methods are available for the isolation of pulmonary alveolar macrophages (Myrvik et al., 1971; Mason et al., 1977), type II alveolar pneumocytes (Kikkawa and Yoneda, 1974; Kikkawa et al., 1975; Mason et al., 1977; Greenleaf et al., 1979; Dobbs et al., 1980) and bronchiolar epithelial Clara cells (Devereux and Fouts, 1980). Up to now, no methods are available for the isolation of alveolar type I pneumocytes or ciliated bronchiolar epithelial cells, which are the lung cells mostly affected during in vivo exposure to oxidative gaseous compounds.

On the next pages the main characteristics of the lung cell lines and primary cultures commonly used for in vitro studies with oxidative gaseous compounds are described.

V79 cell line

V79 cells, originating from Chinese hamster lung fibroblasts, have generally been used to investigate whether a cytotoxic effect could be achieved in a newly developed exposure model. Rasmussen and Crocker (1981), Samuelsen et al. (1978) and Alink et al. (1979) all demonstrated a decreased survival of these cells and a decrease in their colony-forming ability after exposure of the cells in a newly developed and described exposure system.

A549 cell line

The A549 cell line is derived from a human type II alveolar cell carcinoma (Lieber et al., 1976). After exposure of these cells to ozone, inhibition of cell growth, a decrease in plating efficiency and a decrease in viability of the cells have been described (Sweet et al., 1980; Alink et al., 1982). Alink et al. (1980; 1982) and Sweet et al., (1980) also described morphological degenerative changes of these cells after ozone exposure. These included loss of microvilli, surface blebs and vacuole formation.

In addition it was reported that incubation of the A549 cells with

vitamin E provided protection against cytotoxic concentrations of ozone (Alink et al., 1982).

These results indicate that in vitro exposure of cell line cultures provides a model system for the study on mechanisms of toxic action and cellular defense in cells exposed to oxidative gaseous compounds.

Alveolar macrophages

Of all primary lung cell cultures that can be obtained, cultures of alveolar macrophages are most frequently used for in vitro studies on the toxicity of ozone and nitrogen dioxide. This, because they can be isolated from the lungs of experimental animals in a relatively easy way. The functional activity of alveolar macrophages can be represented by their phagocytic and/or bactericidal activity. This parameter has been shown to be impaired after exposure of the cells to both ozone or nitrogen dioxide in vitro (Voisin et al., 1981; Valentine, 1985). The phenomenon is also known to be caused by in vivo exposure (Coffin et al., 1968; Goldstein et al., 1974).

Type II alveolar pneumocytes

Type II alveolar pneumocytes are the lung cells responsible for the production of lung surfactant. Isolated primary cultures of these cells are still capable of synthesizing surfactant lipids. Haagsman et al., (1985) demonstrated effects of ozone on the phospholipid synthesis by isolated type II alveolar pneumocytes. Rietjens et al. (1985) (chapter 6a, this thesis) and Van Bree et al. (in preparation) reported a loss of viability of isolated type II alveolar pneumocytes after in vitro exposure to ozone or nitrogen dioxide. Based on trypan blue exclusion of cells after in vitro exposure to ozone or nitrogen dioxide, alveolar type II cells appeared to be about twice as sensitive towards both oxidative gases as alveolar macrophages (Rietjens et al., 1985; Van Bree et al., in preparation) (chapter 6a + b, this thesis).

Clara cells

Clara cells are known to obtain relatively high levels of xenobiotic metabolizing activity (Boyd, 1977; Boyd et al., 1980).
An appropriate method for isolation of these cells has been described (Devereux and Fouts, 1980), but Rasmussen (1984) concluded in his review that "the behavior of these cells in culture has not been reported, a major problem being the maintenance of sterility during the complicated manipulations involved in the isolation procedure". As a consequence, effects of *in vitro* exposures to ozone or nitrogen dioxide on these bronchiolar non-ciliated Clara cells have not been described up to now.
Upon *in vivo* exposure, variation in the surface characteristics of these cells appeared to be a sensitive indicator of ozone damage (Schwartz et al., 1976; Zitnik et al., 1978), a phenomenon which may be used as a parameter for *in vitro* damage to these cells in the (near) future.

In the present study, 3 of the above mentioned cell types were used: cells of the A549 cell line and primary cultures of alveolar macrophages or type II cells isolated from Wistar rats. When using a cell line one should keep in mind that cells of an established cell line are always dedifferentiated to a certain extent, and may -as a result- have lost some of the characteristics of the cells from which they originate. An example of this can be found in the absence of a detectable amount of glutathione peroxidase activity in cells of the A549 cell line. However, this fact could be exploited when studies were carried out on the role of the glutathione peroxidase catalyzed reaction in the cellular defense against ozone.
The characteristics of primary cultures, on the other hand, will be closer to those of cells *in vivo*, but as they were isolated from rats in the studies presented in this thesis, they have the drawback of not originating from human tissue.

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PART II

EXPERIMENTS

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CHAPTER 4

TOXICITY OF OZONE AND NITROGEN DIOXIDE TO ALVEOLAR MACROPHAGES: A COMPARATIVE STUDY REVEALING DIFFERENCES IN THEIR MECHANISM OF TOXIC ACTION

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ABSTRACT

The toxicity of ozone and nitrogen dioxide is generally ascribed to their oxidative potential. In this study their toxic mechanism of action was compared using an intact cell model. Rat alveolar macrophages were exposed by means of gas diffusion through a teflon film. In this in vitro system ozone appeared to be 10 times more toxic than nitrogen dioxide.

α -Tocopherol protected equally well against ozone and nitrogen dioxide. It was demonstrated that α-tocopherol provided its protection by its action as a radical scavenger and not by its stabilizing structural membrane effect as i) concentrations of α-tocopherol that already provided optimal protection against ozone and nitrogen dioxide did not influence the membrane fluidity of alveolar macrophages and ii) neither one of the structural α-tocopherol analogues tested, i.e.

phytol and the methyl ether of α-tocopherol, could provide a protection against ozone or nitrogen dioxide comparable to the one provided by α-tocopherol.

From these results it was concluded that reactive intermediates, scavenged by α-tocopherol are important in the mechanism of both ozone and nitrogen dioxide induced cell damage. However, further results presented, strongly confirmed that the kind of radicals and/or reactive intermediates, and thus the toxic reaction mechanism involved, must be different in ozone and nitrogen dioxide induced cell damage. This was concluded from the observations which showed that i) vitamin C provided significantly better protection against nitrogen dioxide than against an equally toxic dose of ozone, that ii) glutathione-depletion affected the cellular sensitivity towards ozone to a significantly greater extent than the sensitivity towards nitrogen dioxide and that iii) the scavenging action of α-tocopherol was accompanied by a significantly greater reduction in its cellular level during nitrogen dioxide exposure than during exposure to ozone.

One of the possibilities compatible with the results presented in this study might be the involvement of lipid (peroxyl) free radicals formed in a radical mediated peroxidative pathway, resulting in a substantial break-down of cellular α-tocopherol, in nitrogen dioxide induced cell damage. While ozone induced cell damage might proceed by lipid ozonides, scavenged by α-tocopherol as well.

INTRODUCTION

The toxicity of ozone and nitrogen dioxide is generally ascribed to their oxidative potential. Many observations support a similarity in their toxic mode of action. Both compounds cause similar morphologic changes in the lungs of exposed animals, such as degeneration of type 1 alveolar pneumocytes and loss of ciliated epithelium from the upper airway (Evans et al., 1976; Ibrahim et al., 1980). Besides, both

gaseous compounds have comparable effects on lung cytodynamics as they both cause proliferation of type 2 alveolar pneumocytes and an increase in the number of alveolar macrophages (Goldstein et al., 1974; Mustafa and Tierney, 1978; Zitnik et al., 1978). In addition, ozone and nitrogen dioxide have a similar effect on biochemical lung parameters, i.e. on the activities of antioxidant enzymes (Chow and Tappel, 1973; Sagai et al., 1982; Mustafa et al., 1984). Oxidation of cellular thiol groups or amino acids of functional and structural proteins and/or oxidation of the unsaturated fatty acids in the cell membrane, offers a likely common mechanism of toxic action. However, it is still unknown, whether nitrogen dioxide and ozone exert their toxic action through a common mechanism.

Evidence supporting a dissimilarity in the mechanism of toxic action of ozone and nitrogen dioxide is provided by Goldstein et al. (1977), who demonstrated that agglutination of rat alveolar macrophages by the lectin concanavalin A was influenced by ozone and nitrogen dioxide in an opposite way.

Further evidence for a dissimilarity arises from studies using pure fatty acids or aqueous emulsions of fatty acids (Roehm et al., 1971; Pryor et al., 1976; Srisankar and Patterson, 1979; Pryor et al., 1982). Nitrogen dioxide induced oxidation of unsaturated fatty acids in these model systems proceeds by a radical mediated peroxidative reaction pattern, initiated by either abstraction of an allylic hydrogen atom or by addition of the nitrogen dioxide free radical to the double bond of the fatty acid molecule (Pryor and Lightsey, 1981; Pryor et al., 1982).

On the other hand, the dominant reaction pathway in ozone initiated oxidation of pure fatty acids seems to involve a direct attack of ozone on the fatty acid double bond, followed by the ionair Criegee ozonide mechanism (Criegee, 1957; Roehm et al., 1971; Srisankar and Patterson, 1979). However, when the fatty acids are exposed to ozone in aqueous emulsion, the oxidation proceeds in part by a radical mediated peroxidative reaction pattern as well (Roehm et al., 1971;

Pryor et al., 1976). This indicates that the mechanism(s) occuring in ozone induced oxidation depend on the mode of fatty acid exposure. From these observations it clearly follows that the mechanism by which ozone and nitrogen dioxide actually initiate their oxidative damage to the unsaturated fatty acid moieties in the cell membrane can only be studied adequately by using intact cell models. The latter consideration formed the basis for the present work. In this study, the toxicity of ozone and nitrogen dioxide was compared using an in vitro system in which cell cultures (i.e. rat alveolar macrophages) can be exposed to gaseous compounds by means of gas diffusion through a teflon film (Alink et al., 1979).

The hypotheses formulated to test possible differences or similarities in the mode of toxic action of the compounds can be described as follows. If a certain degree of cell damage caused by ozone and nitrogen dioxide is initiated by the same reaction, i.e. abstraction of an allylic hydrogen atom, the same reactive intermediates will be involved. Such a similarity in reactive initial products must result in a comparable protection provided by cellular antioxidant systems. However, differences in the (initial) toxic reaction pathway, giving rise to different reactive intermediates, will possibly be reflected in a dissimilar protection provided by cellular antioxidant systems.

In the present study, the effect of depletion of cellular glutathione levels on the ozone and nitrogen dioxide sensitivity of alveolar macrophages was compared, as well as the protection provided against equally toxic doses of both gaseous compounds by increased cellular levels of the antioxidant vitamin C and the radical scavenger vitamin E (α-tocopherol).

In addition to these experiments the effect of equally toxic doses of ozone and nitrogen dioxide on the α-tocopherol content of exposed alveolar macrophages was determined. As α-tocopherol is known to be destroyed while acting as an antioxidant in interrupting and terminating the radical mediated peroxidative chain reaction (Fukuzawa et al., 1982), the data obtained in these experiments will provide infor-

mation on the possible involvement of this reaction pathway in the toxic mode of action of ozone or nitrogen dioxide.

Because other reports presented in the literature suggested that the protective action of α-tocopherol results not only from its radical scavenging capacity, but also from the strong physico-chemical association of its phytyl-side chain with arachidonyl moieties of the membrane phospholipids (Lucy, 1972; Diplock and Lucy, 1973; Gutteridge, 1978; Mino and Sugita, 1978), an additional objective of the present study was to investigate whether the protection provided by α-tocopherol should be ascribed to its action as a radical scavenger or to its structural effect on the cell membrane. For this purpose, the effect of protective cellular amounts of α-tocopherol on cell membrane fluidity was determined, as well as the ability of two structural analogues of α-tocopherol, phytol and the methyl ether of α-tocopherol (Figure 1), to provide protection against toxic amounts of ozone or nitrogen dioxide. Results obtained from these experiments will indicate in which way (dis)similarities in protection against ozone and nitrogen dioxide by α-tocopherol, or in cellular α-tocopherol content after exposure to these compounds, should be interpreted.

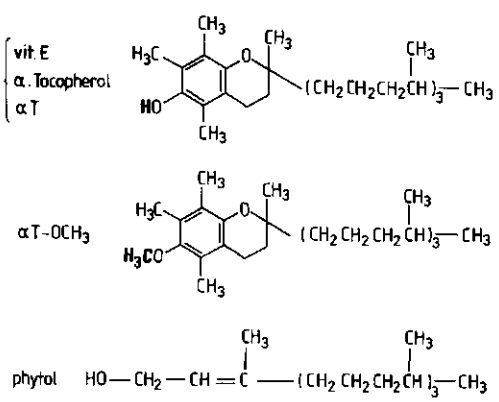

Figure 1. α-Tocopherol and its structural analogues tested in this study.

METHODS

Cell culture

Alveolar macrophages were isolated from the lungs of female Wistar rats (± 200 g) as described by Mason et al. (1977) by the lavage of isolated perfused lungs. Approximately 10^6 cells were plated onto a plastic film dish with a 25 μm teflon bottom and a growth area of 18 cm^2 (Petriperm, Heraeus, Hanau, West-Germany).

Alveolar macrophages were cultured in Ham's F10 medium (Flow, Irvine, Scotland), supplemented with $NaHCO_3$ (1.2 g/l), 10% newborn calf serum (Gibco, Glasgow, Scotland),50 IU/l of penicillin (Gist-Brocades, Delft, The Netherlands), and 50 mg/l streptomycin (Specia, Paris, France) at 37°C in a humid atmosphere containing 5% CO_2 in air. Cells were cultured for 3 days in order to attach to and stretch on the teflon film of the petriperm dish and during these days their cellular antioxidant level(s) could be modified - if desirable - , as described below.

Gas exposure

Cells were exposed to ozone or nitrogen dioxide by means of gas diffusion through the teflon membrane of the petriperm dish (Alink et al., 1979). Before exposure cells were washed and fresh medium without serum was added to the cells.

Ozone was generated by a high voltage discharge type ozone generator (Fisher, Model 501, Meckenheim, West Germany). Nitrogen dioxide (0,9% NO_2 in N_2) was purchased from Matheson (Oevel, Belgium).

The amount of ozone which diffused through the teflon film into the dish was measured using the indigo disulfonic acid method (Guicherit et al., 1972). Nitrogen dioxide was measured using the method described by Huygen (1970) and a nitrite equivalent of nitrogen dioxide, i.e. a Saltzman factor of 0.67.

Modification of cellular antioxidant levels

Alveolar macrophages were depleted of glutathione by treating them with methionine sulfoximine (MSO) (Sigma, St.Louis, USA) and diethylmaleate (DEM) (Riedel-de Haën, Hannover, West Germany) as follows; alveolar macrophages were cultured for 3 days in the presence of 4.0 mM MSO, which is known to inhibit γ-glutamyl-cysteine synthetase (Meister, 1983) - That MSO may also inhibit glutamine synthetase is not of importance, because the glutamine concentration in the medium was 1.0 mM -. Before exposure to ozone or nitrogen dioxide, the cells were preincubated for 2.5 h in the presence of 4.0 mM MSO and 60 μM DEM in a salt solution (sol A) containing 5.5 mM glucose/ 125 mM NaCl/ 5 mM KCl/ 2.5 mM Na_2HPO_4/ 2.5 mM $CaCl_2$/ 1.2 mM $MgSO_4$ and 17 mM HEPES pH 7.4. DEM is known to react with intracellular GSH (Boyland and Chasseaud, 1967). After this preincubation cells were washed and exposed in the presence of sol A without additional MSO or DEM. Incubation of cells with MSO/DEM was shown to reduce cellular glutathione levels significantly (Rietjens et al., 1985).

Cellular levels of α-tocopherol (vitamin E) were increased by culturing the cells in the presence of vitamin E for 2 days. A stock solution of DL-α-tocopherol (Merck, Darmstadt, West Germany) in ethanol (10 mg/ml) was diluted in Ham's F10 medium.

Cellular levels of ascorbic acid (vitamin C) were increased by incubating the cells for 2.5 h at 37°C in the presence of L-ascorbic acid (Merck, Darmstadt, West Germany) in Ham's F10 medium at pH 7.4.

Preincubations of alveolar macrophages with the desired concentrations of the α-tocopherol analogues, phytol (Merck, Darmstadt, West Germany) or α-tocopherol methyl ether (synthesized as described by Cohen et al., 1981), were carried out for 2 days at 37°C in Ham's F10 medium. Stock solutions of these compounds of 10 mg/ml in ethanol were diluted in Ham's F10 medium.

Before exposure to ozone or nitrogen dioxide cells were washed twice and exposed in Ham's F10 without additional serum or antioxidant compound.

Determination of phagocytosis

Phagocytosis was determined after incubation of the cells for 1.5 h at 37°C in the presence of approximately 10^7 dead yeast cells, coloured by boiling them for 30 minutes in a congo red (Fluka, Buchs, Switzerland) PBS solution.

Measurement of fluorescence polarization

Fluorescence polarization was measured essentially as described by De Laat et al. (1977). Approximately $2x10^5$ alveolar macrophages were grown in culture dishes on rectangular glass cover slips. Labelling of these cells was carried out using a 2 μM solution of 1,6-diphenyl-1,3,5-hexatriene (DPH) (Janssen Chimica, Beerse, Belgium) as the fluorescent probe, in sol A instead of PBS, prepared as described by De Laat et al. (1977). Incubation of the cover slips with this DPH solution was carried out at 37°C in the dark for 60 minutes. Fluorescence polarization was measured using a home-made experimental set-up, essentially made as described by De Laat et al. (1977) at 37°C with the cover slips positioned in a quartz cuvette filled with sol A. The fluorescence polarization P was calculated according to equation 1.

$$P = \frac{I_{0/0} - f \times I_{0/90}}{I_{0/0} + f \times I_{0/90}} \qquad -1-$$

with $f = I_{90/0}/I_{90/90}$, the correction factor for intrinsic instrumental polarization.

Determination of cellular α-tocopherol

For determination of cellular levels of α-tocopherol alveolar macrophages were harvested by scraping them from the culture dishes. Cell samples were disrupted by freezing in liquid nitrogen and thawing at

37°C which was repeated twice.

α-Tocopherol was extracted as described by Folch et al. (1957), and measured fluorometrically in ethanol with λ excitation = 295 nm and λ emission = 340 nm (Duggan, 1959).

Statistical analysis of data

Data are presented as mean ± standard error of the mean, and statistical analysis was carried out using the paired Student's t-test, unless indicated otherwise.

RESULTS

The data in Table 1 show that both ozone and nitrogen dioxide caused a dose-dependent decrease in phagocytosis of rat alveolar macrophages. Nitrogen dioxide appeared to be about 10 times less toxic than ozone.

TABLE 1. *EFFECT OF IN VITRO EXPOSURE OF RAT ALVEOLAR MACROPHAGES TO OZONE OR NITROGEN DIOXIDE ON PHAGOCYTOSIS (n=5).*

gas	dose	$\frac{nmol NO_2}{nmol O_3}$	phagocytosis
	in nmol/dish		% of control (=$-O_3$/$-NO_2$)
NO_2	59 ± 3	9.8	78 ± 8
O_3	6 ± 1		81 ± 4
NO_2	97 ± 4	10.8	42 ± 4
O_3	9 ± 1		42 ± 3
NO_2	167 ± 10	9.8	29 ± 3
O_3	17 ± 1		26 ± 3

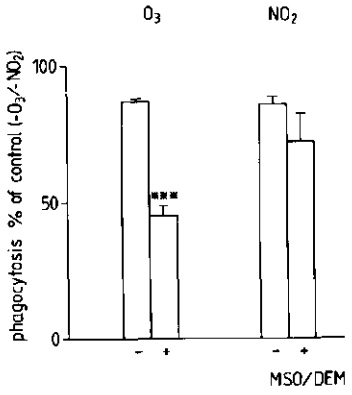

Figure 2. Sensitivity of glutathione-depleted (=MSO/DEM-treated) and untreated alveolar macrophages to ozone (7±1 nmol O_3/dish) and nitrogen dioxide (54±4 nmol NO_2/dish).
*** = $p < 0.001$ (n=5).

The results presented in figure 2 show that depletion of the cellular level of glutathione resulted in a significant increase in the sensitivity of the cells towards ozone, but in a less pronounced increase in their nitrogen dioxide sensitivity. The difference between phagocytic capacity, of untreated and MSO/DEM-treated (= glutathione-depleted) alveolar macrophages amounted to 40 ± 3% after ozone exposure as compared to a significantly ($p < 0.02$) smaller difference amounting to 14 ±8%, after exposure to nitrogen dioxide.

Preincubation of the alveolar macrophages with vitamin C protected the cells against both oxidative gaseous compounds (figure 3), but it is also apparent from these results that vitamin C did provide a significantly better protection against nitrogen dioxide than against an equally toxic dose of ozone.

The protection provided by preincubation of the cells with α-tocopherol appeared to be the same for both ozone and nitrogen dioxide as can be seen from the data presented in figure 4.

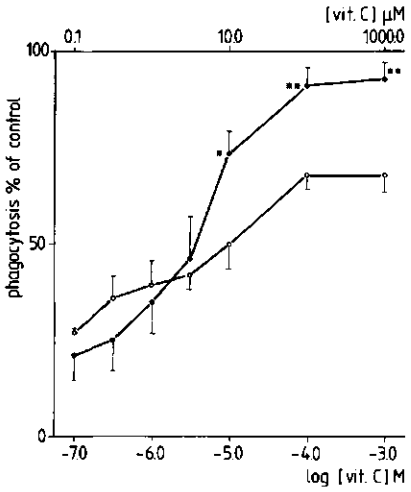

Figure 3. Protection of alveolar macrophages against ozone (17 ± 1 nmol O_3/dish) (-o-) and an equally toxic amount of nitrogen dioxide (177±22 nmol NO_2/dish) (-•-), provided by preincubation of the cells with vitamin C.

* = $p < 0.05$, ** = $p < 0.01$ (unpaired Student's t-test) (n=5).

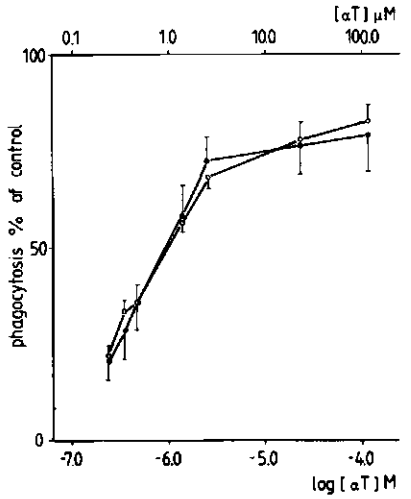

Figure 4. Protection of alveolar macrophages against ozone (14 ± 2 nmol O_3/dish) (-o-) and an equally toxic amount of nitrogen dioxide (172±8 nmol NO_2/dish) (-•-), provided by preincubation of the cells with α-tocopherol. (n=5)

From the results depicted in figure 5 it can be seen that at concentrations of α-tocopherol that already provided optimal protection, no pronounced effect on the cell membrane fluidity was detected. And the data presented in figure 6 a+b clearly demonstrate that neither one of the structural α-tocopherol analogues tested, could provide a protection against ozone or nitrogen dioxide comparable to the protection provided by similar concentrations of α-tocopherol. Both observations indicate that the protective effect of α-tocopherol is not related to a structural effect on the cell membrane.

Finally, the data presented in figure 7 a+b show that equally toxic doses of ozone and nitrogen dioxide did not affect the cellular level of α-tocopherol to the same extent. At doses of both compounds that affected the phagocytosis to the same extent, the percentages of α-tocopherol left in the alveolar macrophages after exposure, differed significantly ($p < 0.01$, unpaired Student's t-test), amounting to 92 ±2% after ozone exposure and to 49 ±12% after exposure to nitrogen dioxide.

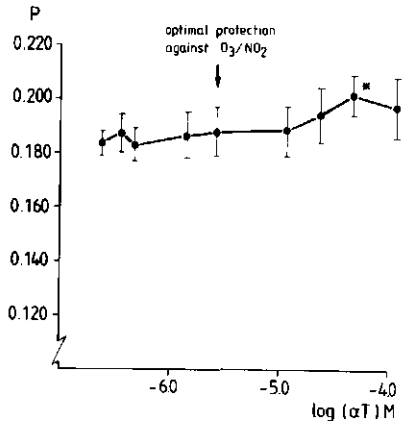

Figure 5. Effect of preincubation with α-tocopherol on the membrane fluidity of alveolar macrophages, measured as the degree of fluorescence polarization (P) of 1,6-diphenyl-1,3,5-hexatriene (DPH).
* = $p < 0.05$ (n=5).

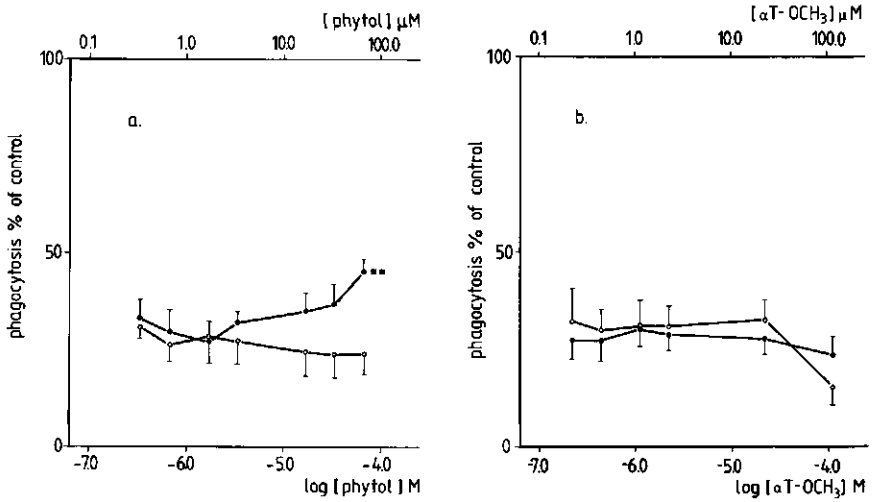

Figure 6. Effect of preincubation of alveolar macrophages with phytol **(a)** or the methylether of α-tocopherol **(b)** on their sensitivity towards ozone (-o-) and nitrogen dioxide (-●-).
The ozone doses amounted to respectively 19±1 nmol O_3/dish **(a)** and 16±1 nmol O_3/dish **(b)**. The doses of nitrogen dioxide amounted to 158±7 nmol NO_2/dish **(a)** and 163±8 nmol NO_2/dish **(b)**.
** = $p < 0.01$ (unpaired Student's t-test) (n=5).

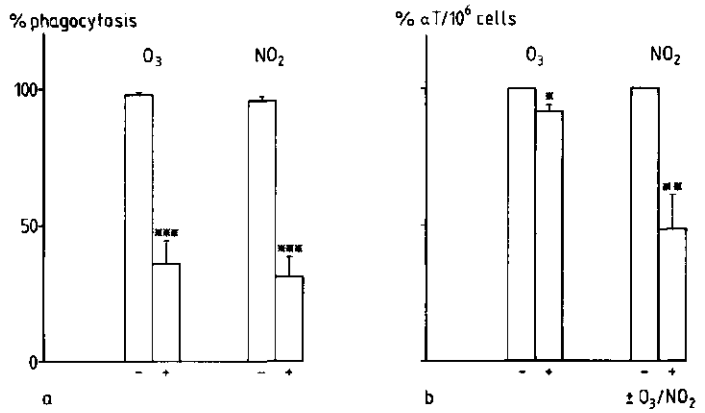

Figure 7. Effect of ozone (21±1 nmol O_3/dish) and nitrogen dioxide (271±11 nmol NO_2/dish) on **(a)** phagocytosis and **(b)** α-tocopherol content of alveolar macrophages. Alveolar macrophages were incubated in the presence of 23 µM α-tocopherol for 2 days, washed and exposed to ozone or nitrogen dioxide in the presence of only 1 ml Ham's F10 without additional serum or α-tocopherol.
* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ (n=5).

DISCUSSION AND CONCLUSIONS

In the exposure model used ozone appeared to be about 10 times more toxic than nitrogen dioxide. Other *in vitro* studies described in the literature reported ozone to be more toxic than nitrogen dioxide by a factor ranging from 1 (Voisin et al., 1980) to ± 3000 (Weissbecker et al., 1969), probably depending on the exposure model applied. The *in vitro* system used in the present study yields a difference in ozone and nitrogen dioxide toxicity comparable to the difference reported in *in vivo* studies, in which the factor amounted to 10 to 25 (Stockinger, 1965; Chow et al., 1974; Goldstein et al., 1974; Bils, 1974; Evans et al., 1976).

Furthermore, the amount of oxidant gas in nmol/dish applied in our *in vitro* system can be compared to an *in vivo* exposure using the model for pulmonary deposition of ozone, presented by Miller et al. (1978). Using this model it can be calculated that at a tracheal ozone concentration of 0.1–0.5 ppm, with 10–20 breaths per minute, the ozone dose to which lung cells would be exposed during 2.5 h varies between 2–23 nmol ozone/18 cm^2 (= growth area of the petriperm dish)/2.5h, which is the range actually applied in our *in vitro* system.

It was shown that preincubation of the cells with vitamin C provided protection during a subsequent exposure to either ozone, or nitrogen dioxide. Comparison of the protection provided against doses which affected the phagocytosis to the same extent, revealed that vitamin C provided significantly better protection against nitrogen dioxide than against ozone. These results indicate that the mechanisms of cellular damage caused by ozone and nitrogen dioxide do not proceed by the same reactive intermediates.

Additional evidence for a different cellular mechanism of toxic action in ozone and nitrogen dioxide induced cell damage, follows from the observation that depletion of the cellular glutathione level affected

the ozone sensitivity of the alveolar macrophages to a significantly greater extent than their sensitivity towards nitrogen dioxide.

In contrast to the difference between nitrogen dioxide and ozone toxicity demonstrated by glutathione depleted or vitamin C enriched alveolar macrophages, protection by α-tocopherol did not reveal a different pattern of protection for ozone and nitrogen dioxide exposed cells.

The most likely explanations for this discrepancy are that **i)** the membrane-bound α-tocopherol functions at another cell-site than the cytoplasmatic antioxidants vitamin C and glutathione, or **ii)** that α-tocopherol accomplishes its protection mainly by means of its structural, stabilizing effect on the cell membrane (Lucy, 1972; Diplock and Lucy, 1973; Maggio et al., 1977; Gutteridge, 1978; Mino and Sugita, 1978; Diplock, 1982), and not by its action as a radical scavenger.

However, the latter explanation is contradicted by the observations showing that protective concentrations of α-tocopherol did not influence cell membrane fluidity and that neither one of the structural α-tocopherol analogues tested, phytol or the methyl ether of α-tocopherol, could offer a pronounced cellular protection against ozone or nitrogen dioxide. As it is clearly demonstrated by these results that the protective action of α-tocopherol is mediated by means of its scavenging capacity, provided by its 6-hydroxyl group, the observation that α-tocopherol protected equally well against ozone and nitrogen dioxide leads to the evident conclusion that reactive intermediates scavenged by α-tocopherol are important in the mechanism of both ozone and nitrogen dioxide induced cell damage. But from the facts that **i)** vitamin C provided different protection against both gaseous compounds and that **ii)** glutathione depletion affected the cellular sensitivity towards ozone to a greater extent, it follows that the kind of radicals and/or reactive intermediates formed, and therefore the reaction mechanism involved must be different in ozone and nitrogen dioxide induced cell damage. Finally this conclusion is also strongly con-

firmed by the observation which showed that **iii)** doses of ozone and nitrogen dioxide which equally affected the phagocytosis of the alveolar macrophages, resulted in a dissimilar break-down of the α-tocopherol in the exposed cells.

One of the possibilities compatible with the results presented in this study might be the involvement of lipid (peroxyl) free radicals formed in a radical mediated peroxidative reaction pathway, which causes a substantial break-down of cellular α-tocopherol, in nitrogen dioxide induced cell damage. While ozone induced cell damage might proceed by lipid ozonides, scavenged by α-tocopherol as well.

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CHAPTER 5

INFLUENCE OF POLYUNSATURATED FATTY ACID SUPPLEMENTATION AND MEMBRANE FLUIDITY ON OZONE AND NITROGEN DIOXIDE SENSITIVITY OF RAT ALVEOLAR MACROPHAGES

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ABSTRACT

The phospholipid polyunsaturated fatty acid (PUFA) content and the membrane fluidity of rat alveolar macrophages were modified dose dependently and in different ways. This was done to study the importance of both membrane characteristics for the cellular sensitivity towards ozone and nitrogen dioxide. Cells preincubated with arachidonic acid (20:4) complexed to bovine serum albumin (BSA) demonstrated an increased *in vitro* sensitivity versus ozone and nitrogen dioxide. The phenomenon was only observed at the highest 20:4 concentrations tested, whereas the membrane fluidity of the 20:4 treated cells al-

ready showed a maximum increase at lower preincubation concentrations. Hence it could be concluded that the increased ozone and nitrogen dioxide sensitivity of PUFA enriched cells is not caused by their increased membrane fluidity, resulting in an increased accessibility of sensitive cellular fatty acid moieties or amino acid residues. This conclusion receives further support from the observation that a comparable increase in membrane fluidity without a concomitant increase in PUFA content, achieved by preincubation of the cells with egg lecithin liposomes, did not result in an increased sensitivity of the cells towards ozone or nitrogen dioxide.

Analysis of the fatty acid composition of PUFA enriched cells demonstrated that - in contrast to membrane fluidity - the PUFA (especially 20:4) content of the cells continued to increase at the 20:4 preincubation concentrations increasing from 33 to 150 μM.

This indicated that the increased ozone and nitrogen dioxide sensitivity of PUFA enriched alveolar macrophages should be ascribed to their increased percentage of sites at which cell damage may start and/or proceed.

These results strongly support the involvement of lipid oxidation in the mechanism(s) of toxic action of both ozone and nitrogen dioxide in an intact cell system.

INTRODUCTION

Ozone and nitrogen dioxide are the major oxidants of photochemical air pollution. Their effects on morphological, functional, physiological and biochemical lung parameters are well known (e.g. Menzel, 1984; Morrow, 1984).

The mechanism of action of both compounds is generally ascribed to oxidation of sensitive amino acid residues in cellular proteins and/or oxidation of unsaturated fatty acid moieties in membrane phospholipids. The potential of ozone and nitrogen dioxide to react with

unsaturated fatty acids is demonstrated by the identification of fatty acid oxidation products after exposure of pure fatty acid monolayers or suspensions (Roehm et al., 1971; Srisankar and Patterson, 1979; Pryor et al., 1982). Existing evidence for the involvement of lipid (per)oxidation in the mechanism of action of ozone and/or nitrogen dioxide *in vivo* appears to depend on three lines of evidence: **i)** the occurrence of products of lipid peroxidation in the breath, lung homogenates or isolated lung lipids of exposed animals. Examples of this can be found in the presence of thiobarbituric acid reactive products in lung homogenates of rats exposed to nitrogen dioxide (Sagai et al., 1982), the demonstration of increased diene conjugation in lung lipids extracted from nitrogen dioxide or ozone exposed rodents (Thomas et al., 1968; Goldstein et al., 1969) and the detection of increased amounts of pentane in the breath of ozone exposed, vitamin E deficient rats (Dumelin et al., 1978a; Tappel and Dillard, 1981) or of ethane in the breath of rats exposed to nitrogen dioxide (Sagai et al., 1981; Sagai et al., 1982).

ii) The second line of evidence refers to experiments demonstrating changes in the lung lipid composition of ozone and nitrogen dioxide exposed animals (Menzel et al., 1978; Kobayashi et al., 1980).

iii) The third line of evidence refers to the protection offered by the antioxidant vitamin E (Fletcher and Tappel, 1973; Chow et al., 1979; Sato et al., 1976).

However, for a number of reasons the evidence is not really conclusive. So far, for instance, any relation with dose or level of exposure has not been demonstrated for most of these phenomena. In addition the detection of thiobarbituric acid reactive material may be non-specific as some compounds and/or reactions are known to interfere with the assay (Gray, 1978). Moreover detection of conjugated dienes by ultraviolet spectroscopy may not be specific as well (Logani and Davies, 1980; Waller and Recknagel, 1977; Menzel et al., 1975).

Besides, an increased diene conjugation in extracted lung lipids could only be demonstrated several hours after exposure to the oxidative

compounds (Thomas et al., 1968). It is also noteworthy that increased amounts of pentane could not be detected in the breath of **i)** ozone exposed monkeys, **ii)** ozone exposed rats fed a vitamin E containing commercial diet, or **iii)** vitamin E deficient rats exposed to nitrogen dioxide (Dumelin et al., 1978a; Dumelin et al., 1978b; Dillard et al., 1980).

Furthermore, modifications in lung lipid composition after exposure to ozone or nitrogen dioxide can also be explained by changes in the lung cell populations resulting from proliferation and/or destruction of certain lung cell types, or by changes in lipid metabolism of individual lung cells (Kobayashi et al., 1980).

In summary, the evidence referred to is not conclusive. The strongest support available for the involvement of lipid (per)oxidation in the mechanism of action of both ozone and nitrogen dioxide is of an indirect nature, viz. the protection provided by the antioxidant action of vitamin E (Fletcher and Tappel, 1973; Chow et al., 1979; Sato et al., 1976).

The exposure of isolated or cultured cells seems to represent an adequate model for studying the involvement of lipid oxidation in the mechanism of toxic action of ozone and nitrogen dioxide. An example can be found in a recent paper of Konings (1986) who reported that mouse fibroblast LM cells showed an increased sensitivity towards ozone after enrichment of their polyunsaturated fatty acid (PUFA) membrane moieties.

In theory, PUFA enrichment may influence cellular sensitivity in two ways. First, PUFA enriched membranes contain an increased number of sites at which cell damage could start and/or proceed. In this case, the increased ozone sensitivity of PUFA enriched cells would point to an involvement of lipid (per)oxidation in the mechanism of ozone toxicity.

Secondly, it can be suggested that an increased sensitivity of PUFA enriched cells could be caused by their increased membrane fluidity. As a result the membranes and/or the cytoplasm of PUFA enriched cells might become more accessible and exposed.

The objective of the present study was to investigate the possible contribution of either one of these hypothetical mechanisms with regard to the increased sensitivity of PUFA enriched cells versus both ozone and nitrogen dioxide. The study was carried out with an in vitro model in which cells can be exposed to gaseous compounds by means of gas diffusion through a thin teflon film (Alink et al., 1979; Rietjens et al., 1986). To study the relative contribution of membrane fluidity and PUFA content on cellular sensitivity towards ozone and nitrogen dioxide, membrane fluidity was modified dose dependently and in two ways. First by preincubation of the cells with arachidonic acid (20:4) complexed to bovine serum albumin (BSA) and secondly by preincubation of the cells with egg lecithin liposomes.

The first procedure was expected to increase cellular membrane fluidity by increasing the percentage of polyunsaturated fatty acid moieties (Konings, 1986; Shinitzky and Barenholz, 1978). The second method was expected to increase membrane fluidity without a pronounced increase of the cellular PUFA content, as egg lecithin is known to increase membrane fluidity by increasing the ratio lecithin:sphingomyelin of the membrane and/or by extracting native cholesterol from the cell surface membranes (Shinitzky and Barenholz, 1978; Shinitzky and Inbar, 1974).

METHODS

Cell culture

Alveolar macrophages were isolated from the lungs of female Wistar rats (± 200 g) as described by Mason et al. (1977) by the lavage of perfused lungs. Approximately 10^6 cells were plated onto a plastic film dish with a 25 µm teflon bottom and a growth area of 18 cm^2 (Petriperm, Heraeus, Hanau, West Germany). Alveolar macrophages were cultured in Ham's F10 medium (Flow, Irvine, Scotland), supplemented with $NaHCO_3$ (1.2 g/l), 10% newborn calf serum (Gibco, Glasgow,

Scotland), 50 IU/l of penicillin (Gist-Brocades, Delft, The Netherlands), and 50 mg/l streptomycin (Specia, Paris, France) at 37°C in a humid atmosphere containing 5% CO_2 in air. Cells were cultured for 3 days in order to attach to and stretch on the teflon film of the petriperm dish before they were exposed to ozone or nitrogen dioxide, and during these days their membrane fluidity could be modified as described below.

Gas exposure

Cells were exposed to ozone or nitrogen dioxide by means of gas diffusion through the teflon membrane of the petriperm dish (Alink et al., 1979). Before exposure cells were washed and fresh medium without serum was added to the cells.

Ozone was generated by a high voltage discharge type ozone generator (Fisher, Model 501, Meckenheim, West Germany). Nitrogen dioxide (0.9% NO_2 in N_2) was purchased from Matheson (Oevel, Belgium). The amount of ozone which diffused through the teflon film into the dish was measured using the indigo disulfonic acid method (Guicherit et al., 1972). Nitrogen dioxide was measured using the method described by Huygen (1970) and a nitrite equivalent of nitrogen dioxide, i.e. a Saltzman factor of 0.67.

Measurement of membrane fluidity

Membrane fluidity was measured as the degree of fluorescence polarization (P) of 1,6-diphenyl-1,3,5-hexatriene (DPH) (Janssen Chimica, Beerse, Belgium). Fluorescence polarization was measured essentially as described by De Laat et al. (1977).

Approximately 2×10^5 alveolar macrophages were grown in culture dishes on rectangular glass cover slips. Labelling of these cells was carried out using a 2 μM solution of DPH in a salt solution (sol A) containing 5.5 mM glucose/125 mM NaCl/5 mM KCl/2.5 mM Na_2HPO_4/2.5 mM $CaCl_2$/1.2 mM $MgSO_4$ and 17 mM HEPES pH 7.4, instead of PBS, and prepared as described by De Laat et al. (1977). Incubation of the cover

slips with this DPH solution was carried out at 37°C in the dark for 60 minutes. Fluorescence polarization was measured using a self-constructed experimental set up, essentially made as described by De Laat et al. (1977), at 37°C, with the cover slips positioned in a quartz cuvette filled with sol A. The degree of fluorescence polarization P was calculated according to

$$P = \frac{I_{0/0} - f \times I_{0/90}}{I_{0/0} + f \times I_{0/90}}$$

with $f = I_{90/0}/I_{90/90}$, the correction factor for intrinsic instrumental polarization.

Modification of cellular membrane fluidity

Cellular membrane fluidity of alveolar macrophages was modified by preincubation of the cells with arachidonic acid (20:4) (Sigma, St. Louis M.O., U.S.A. Grade I) complexed to fatty acid free bovine serum albumin (BSA) (Sigma, St. Louis, M.O., U.S.A.) or with egg lecithin-(Merck, Darmstadt, West Germany) liposomes.

For BSA/20:4 modification, alveolar macrophages were incubated for 18 hours at 37°C and 5% CO_2, with desired dilutions - in Ham's F10 medium without additional serum -, of a stock solution of 1000 µM 20:4 complexed to 250 µM BSA, prepared as described by Spector and Hoak (1969) and Wolters and Konings (1982).

Modification of membrane fluidity by egg lecithin was carried out by incubating the alveolar macrophages for 2.5 hours at 37°C, 5% CO_2 with desired concentrations of lecithin liposomes in Ham's F10. Lecithin liposomes were prepared as follows. A solution of 100 mg egg lecithin in 3 ml chloroform:methanol (2:1) was evaporated to dryness under nitrogen, dispersed in Ham's F10 to a final concentration of 5 mg/ml and sonicated for 5 minutes with a Branson Sonic Power Sonicator at maximum energy output. Fresh liposomes were prepared for each experiment. Lecithin concentrations, expressed in mg/ml were converted to

molar concentrations using the molecular weight value of 762, determined by Diplock et al. (1977). This could be done as the fatty acid composition of the egg lecithin used in these experiments agreed very well with the fatty acid composition of the egg lecithin used by Diplock et al. (1977). Before exposure to ozone or nitrogen dioxide cells were washed twice and exposed in Ham's F10 medium without additional serum, BSA/20:4 or egg lecithin.

Fatty acid analysis

Lipid extraction from cellular fractions was done according to Konings (1970) and followed by transesterification of the fatty acids carried out as reported by Konings et al. (1980). Fatty acid methyl esters were identified by capillary GLC (Muskiet et al., 1983) using a Packard Gaschromatograph Model 429 equipped with a flame ionization detector and integrator system (Packard, Becker, Delft, The Netherlands).

The column used was a fused silicagel capillary column type DB-5, with a length of 30 meter and an internal diameter of 0.25 mm. Helium was used as the carrier gas. Heating was performed at an initial temperature of 155°C followed by an increase of 2.5°C/min for 30 minutes and then 10°C/min up to 300°C.

Fatty acid methyl esters were identified on the basis of comparison of their retention times to the retention times of different standards.

Determination of phagocytosis

Phagocytosis was determined after incubation of the cells for 1.5 h at 37°C in the presence of approximately 10^7 dead yeast cells coloured by boiling them for 30 minutes in a congo red (Fluka, Buchs, Switzerland) PBS solution.

Statistical analysis of data

Data are presented as mean $\pm$ standard error of the mean, and statistical analysis was carried out using the unpaired Student's t-test, unless indicated otherwise.

RESULTS

The data presented in Figure 1 show that the membrane fluidity of alveolar macrophages, measured as the degree of fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene, can be increased (P value decreased) in a dose dependent way by preincubation of the cells with BSA/20:4 (Figure 1a) or with egg lecithin liposomes (Figure 1b). Incubation of the cells with BSA alone did not influence membrane fluidity of the alveolar macrophages (Figure 1a). In addition Figure 1 demonstrates that egg lecithin and BSA/20:4 can cause a comparable increase in membrane fluidity.

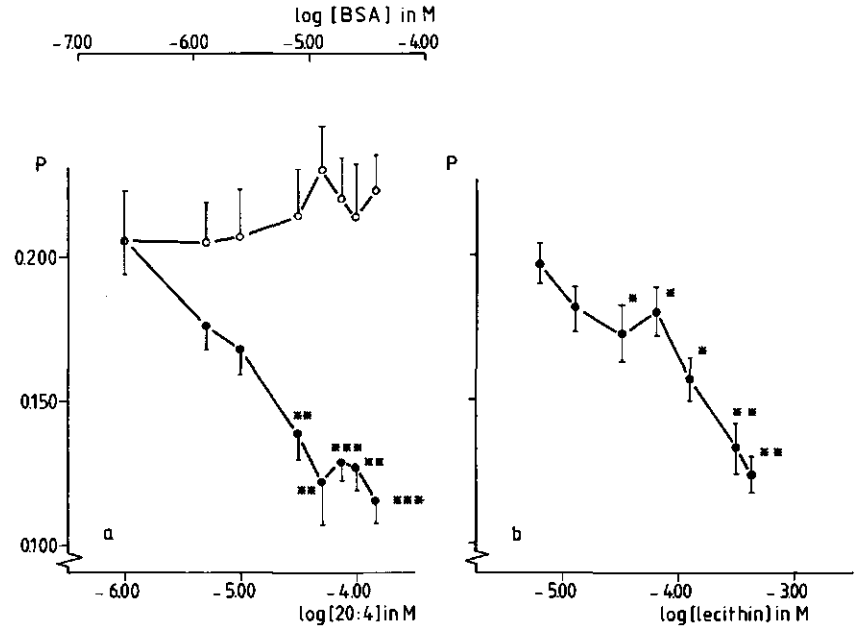

Figure 1. Effect of preincubation with a) BSA/20:4 (- • -) and BSA (- o -) or with b) egg lecithin, on the membrane fluidity of alveolar macrophages, measured as the degree of fluorescence polarization (P) of 1,6-diphenyl-1,3,5-hexatriene (DPH).
* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ ($n = 5$)
b) paired Student's t-test.

From the results presented in Table 1 it follows that the PUFA content of the membrane phospholipids increased in a dose dependent way with the BSA/20:4 concentration. This increase is entirely caused by an increase in the percentage of 20:4 moieties. BSA alone did not influence the fatty acid composition of alveolar macrophages (Table 1). The data depicted in Table 2 show that preincubation of the cells with egg lecithin resulted in a dose dependent decrease in the percentage of phospholipid PUFA moieties. Thus, preincubation with egg lecithin liposomes results in an increased cellular membrane fluidity without a concomitant increase in membrane PUFA content.

TABLE 1 FATTY ACID COMPOSITION OF THE PHOSPHOLIPIDS OF ALVEOLAR MACROPHAGES PREINCUBATED WITH INCREASING CONCENTRATIONS OF BSA/20:4.
control : $[20:4] = 0$; BSA control : $[BSA] = 37.5$ µM
ϕ = fatty acid percentage unchanged with increasing $[20:4]$,
$\uparrow$ = increased, $\downarrow$ = decreased (n = 2-3)

Fatty acid[1)]	control	log $[20:4]$ in M/ $[20:4]$ in µM -4.5/33	-4.3/50	-3.8/150		BSA control
14:0	0.6 ± 0.1	1.2 ± 0.5	0.7 ± 0.1	0.9 ± 0.4	ϕ	1.8 ± 0.6
16:0	16.6 ± 0.1	17.5 ± 1.6	16.1 ± 2.4	15.6 ± 2.6	ϕ	14.1 ± 0.6
16:1 ω7	1.8 ± 0.1	1.6 ± 0.1	1.8 ± 0.1	1.4 ± 0.1	ϕ	2.5 ± 0.4
18:0	17.7 ± 0.9	17.9 ± 0.4	16.6 ± 0.6	16.7 ± 1.7	ϕ	15.6 ± 0.1
18:1 ω7	2.7 ± 0.3	2.3 ± 0.3	1.9 ± 0.4	1.5 ± 0.1	$\downarrow$	3.8 ± 0.3
18:1 ω9	14.9 ± 0.8	9.8 ± 0.2	9.8 ± 0.4	7.7 ± 0.2	$\downarrow$	14.0 ± 0.7
18:2 ω6	3.2 ± 0.1	1.9 ± 0.2	1.7 ± 0.1	1.6 ± 0.2	$\downarrow$	3.7 ± 0.6
20:4 ω6	18.0 ± 0.3	27.5 ± 1.4	33.0 ± 1.0	39.6 ± 2.2	$\uparrow$	16.9 ± 2.2
22:4 ω6	10.0 ± 0.2	13.1 ± 1.0	12.0 ± 1.6	9.9 ± 1.6	ϕ	11.0 ± 0.6
22:5 ω3	7.3 ± 0.5	4.0 ± 0.6	3.2 ± 0.7	2.7 ± 0.6	$\downarrow$	9.5 ± 0.1
22:5 ω6	1.6 ± 0.1	0.9 ± 0.2	1.0 ± 0.4	0.9 ± 0.3	$\downarrow$	1.5 ± 0.1
22:6 ω3	5.8 ± 0.1	2.4 ± 0.5	2.2 ± 0.7	1.4 ± 0.2	$\downarrow$	5.6 ± 0.4
% PUFA	45.9 ± 0.1	49.8 ± 1.6	53.1 ± 2.3	56.1 ± 5.0	$\uparrow$	48.2 ± 2.7

[1)] number of carbon atoms: number of double bonds followed by the position of the double bonds.

TABLE 2 FATTY ACID COMPOSITION OF THE PHOSPHOLIPIDS OF ALVEOLAR MACROPHAGES PREINCUBATED WITH INCREASING CONCENTRATIONS OF EGG LECITHIN.

ϕ = fatty acid percentage unchanged with increasing [egg lecithin]

↑ = increased, ↓ = decreased (n = 2)

Fatty acid[1)]	log [egg. lecithin] in M			
	-5.2	-3.9	-3.4	
14:0	3.6 ± 0.5	4.1 ± 0.8	2.3 ± 0.4	ϕ
15:0	1.6 ± 0.2	1.7 ± 0.4	0.5 ± 0.5	ϕ
16:0	17.0 ± 1.9	17.9 ± 0.8	24.4 ± 0.1	↑
16:1 ω7	3.9 ± 0.1	3.7 ± 0.2	2.8 ± 0.3	ϕ
18:0	10.5 ± 3.0	13.8 ± 1.4	17.2 ± 1.8	↑
18:1 ω7	2.1 ± 0.3	1.0 ± 1.0	1.7 ± 0.1	ϕ
18:1 ω9	28.8 ± 2.6	31.0 ± 4.6	29.5 ± 2.5	ϕ
18:2 ω6	13.2 ± 1.2	13.7 ± 2.5	13.0 ± 1.4	ϕ
20:4 ω6	8.9 ± 3.5	9.9 ± 2.2	6.0 ± 0.6	ϕ
22:4 ω6	8.8 ± 5.7	1.7 ± 1.6	1.5 ± 0.2	↓
22:5 ω3	0.8 ± 0.8	0.7 ± 0.7	0.5 ± 0.5	ϕ
22:6 ω3	0.6 ± 0.6	0.8 ± 0.8	0.7 ± 0.7	ϕ
% PUFA	32.3 ± 2.0	26.8 ± 2.8	21.7 ± 0.7	↓

1) number of carbon atoms: number of double bonds, followed by the position of the double bonds.

Figure 2 shows that preincubation of the alveolar macrophages with lecithin did not influence their sensitivity towards ozone or nitrogen dioxide. However, preincubation of the alveolar macrophages with BSA/20:4 resulted in a dose dependent increase in the ozone as well as the nitrogen dioxide sensitivity of the cells as compared to BSA treated control populations (Figure 3). The difference in phagocytosis of BSA and BSA/20:4 treated alveolar macrophages after ozone exposure amounted to 8 ± 5% and 19 ± 5% ($p < 0.001$) at 100 and 150 µM 20:4 respectively, whereas after nitrogen dioxide exposure these differences amounted to 15 ± 7% ($p < 0.05$) and 26 ± 8% ($p < 0.01$) respectively.

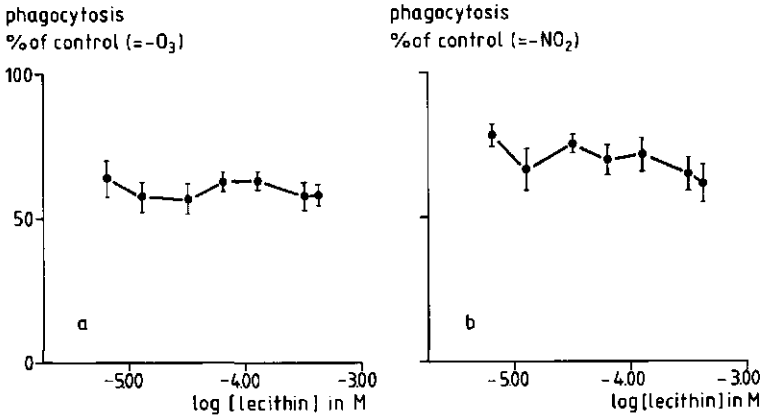

Figure 2. Effect of preincubation with egg lecithin on a) ozone and b) nitrogen dioxide sensitivity of alveolar macrophages (n = 8).
The ozone dose amounted to 7 ± 1 nmol O_3/dish, the nitrogen dioxide dose to 55 ± 7 nmol NO_2/dish.

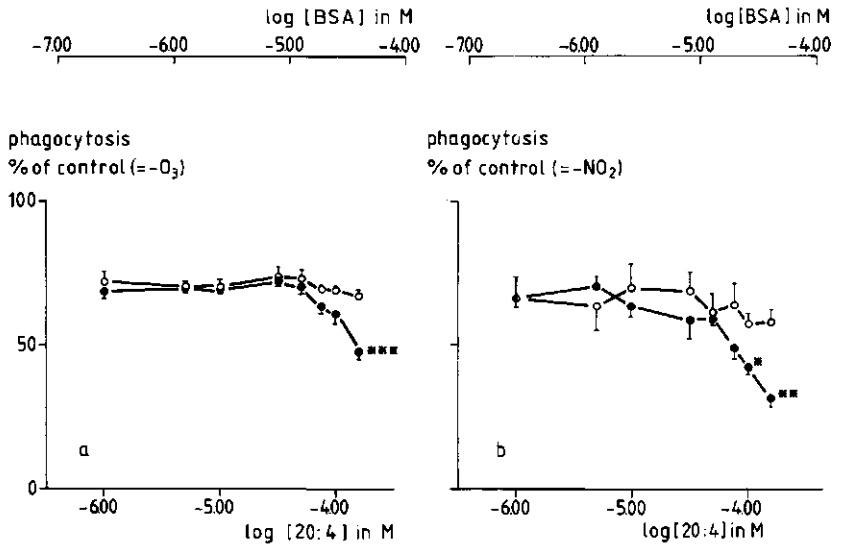

Figure 3. Effect of preincubation with BSA/20:4 (- • -) or BSA (- o -) on a) ozone and b) nitrogen dioxide sensitivity of alveolar macrophages.
The ozone doses amounted to 26 ± 1 (- • -) nmol O_3/dish and 29 ± 1 (- o -) nmol O_3/dish.
The nitrogen dioxide doses amounted to 293 ± 19 (- • -) nmol NO_2/dish and 263 ± 12 (- o -) nmol NO_2/dish.
* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ (n = 5-7).

DISCUSSION AND CONCLUSIONS

Results presented in this study clearly demonstrate the increased sensitivity of PUFA enriched (= BSA/20:4 treated) rat alveolar macrophages towards both ozone and nitrogen dioxide.

This observation provides additional support to the earlier findings reported by Konings (1986) who found an increased sensitivity of PUFA enriched mouse lung fibroblast LM cells versus ozone. Donovan et al. (1977) found that elevation of lung PUFA levels in mice fed a high PUFA diet had little effect on the mortality rate of the animals when they were exposed to ozone. May be this discrepancy relates to the fact that PUFA levels of whole lung tissue can not be modified to the same extent as the PUFA content of isolated or cultured cells modified in vitro.

Further results presented in the present study also demonstrate that the increased sensitivity of PUFA enriched cells should be ascribed to an increased number of sensitive sites at which cell damage may start and/or proceed, and not to an increased membrane fluidity. This conclusion is based on the following results.

First, the increased ozone and nitrogen dioxide sensitivity of the PUFA enriched alveolar macrophages only appeared when cells had been preincubated with 20:4 concentrations of 100 or 150 μM. This in spite of the fact that the maximum increase in membrane fluidity - presented by a maximum decrease of the fluorescence polarization of the DPH probe - was already achieved at the lower preincubation concentrations of 50 and 75 μM.

In addition, the absence of an involvement of increased membrane fluidity in the mechanism of increased ozone and nitrogen dioxide sensitivity also finds support from the observation that a comparable increase in membrane fluidity, without a concomitant increase in cellular PUFA content, achieved by preincubation of the cells with egg lecithin, did not result in an increased sensitivity of the cells.

Finally, the results show that - in contrast to membrane fluidity - the 20:4 and PUFA content of the cells continued to increase at 20:4 concentrations increasing from 33 to 150 μM, which clearly indicates that the increased sensitivity of PUFA enriched cells should be ascribed to an increased content of sensitive fatty acids.

A separate finding of the present study is the fact that the increased sensitivity of PUFA enriched alveolar macrophages showed a tendency to be more pronounced with nitrogen dioxide than with ozone exposure. Such a difference would point to a difference in their respective mechanisms of toxic action, a phenomenon demonstrated more clearly in a previous report by differences in antioxidant protection and α-tocopherol destruction in alveolar macrophages exposed to equally toxic amounts of ozone or nitrogen dioxide (Rietjens et al., 1986).

The results reported in the present study provide additional insight into the mechanism by which ozone and nitrogen dioxide exert their toxic effects as they strongly support the involvement of lipid oxidation in the mechanism by which damage is induced in an intact cell system.

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CHAPTER 6a

GLUTATHIONE PATHWAY ENZYME ACTIVITIES AND THE OZONE SENSITIVITY OF LUNG CELL POPULATIONS DERIVED FROM OZONE EXPOSED RATS

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SUMMARY

Rats were exposed to 1.5 ± 0.1 mg ozone/m^3 for 4 days and the activities of glucose-6-P dehydrogenase (G6PDH), glutathione reductase (GR) and glutathione peroxidase (GSHPx) were measured in the cytosolic fraction of lungs from exposed and control rats. Enzyme activities were also measured in isolated alveolar macrophages and type II cells. After ozone exposure enzyme activities, expressed per gram of protein, showed the following changes. G6PDH activity was increased ($P < 0.001$) in the whole rat lung and showed the same tendency in isolated alveolar macrophages and type II cells. GR activity did not change significantly neither in whole lungs, nor in isolated cell populations. GSHPx activity was increased ($P < 0.001$) in whole lung homogenates, and was also markedly increased in both alveolar macrophages ($P < 0.05$) and type II cells ($P < 0.01$) isolated from ozone-exposed rats. From these results it was concluded that biochemical changes measured in whole lung homogenates might reflect biochemical changes that occur within specific cell types. Furthermore, it was demonstrated, using an in vitro ozone exposure system, that lung cell populations isolated from ozone-exposed rats, in spite of their marked increase in GSHPx activity, did not show a decreased ozone sensitivity compared to cells from unexposed rats, as determined by trypan blue exclusion or phagocytosis. So an increase in GSHPx activity might not be related to an increased cellular resistance to ozone.

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Abbreviations: G6PDH, glucose-6-P-dehydrogenase; GR, glutathione reductase; GSHPx, glutathione peroxidase.

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Key words: Ozone; Glucose-6-phosphate dehydrogenase; Glutathione reductase; Glutathione peroxidase; Resistance; Type II cells; Alveolar macrophages

INTRODUCTION

Ozone is the major oxidant of photochemical smog. Its toxicity has been ascribed to several reactions, including peroxidation of unsaturated fatty acids in the cell membrane [1]. In addition, various physiological, morphological and biochemical changes of the lung have been described following exposure to this air pollutant [1]. Some of these changes have been related to adaptation, a phenomenon well documented for animals [1] and man [2]. Different possibilities have been proposed to explain the observed decreased sensitivity after an initial exposure to a toxic but non-lethal dose of ozone [1,3]. One of the mechanisms involved in protection against ozone toxicity might be the enhancement of the activity of enzymes of the socalled glutathione pathway, which are supposed to reduce toxic fatty acid hydroperoxides, giving rise to the corresponding alcohols [4]. Evidence for this hypothesis, however, appears to be indirect, for it is primarily based on the observation that the activities of glutathione peroxidase (GSHPx), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase of rat lungs increased above their respective controls after several days of exposure of rats to ozone [4]. Furthermore, morphological changes like replacement of type I epithelial cells by less sensitive type II cells, might be related to the increased resistance against ozone toxicity [1,5]. Proliferation or migration of alveolar macrophages might contribute to a decreased ozone sensitivity as well.

Because of these morphological changes it is not known whether the biochemical changes in the lungs of ozone-exposed rats, should be attributed to intrinsic cellular metabolic changes and/or migration, proliferation or replacement of certain cell types in the lung. Therefore, we decided to investigate whether cell populations isolated from the lungs of ozone-exposed rats showed changes in G6PDH, GR and GSHPx activity comparable to those observed in the whole lungs. Secondly, it was investigated whether cells isolated from ozone-exposed rats showed a decreased sensitivity towards ozone after exposure in vitro. In this way more insight might be obtained in the role of enzymes belonging to the glutathione system and the hexose monophosphate shunt, in the cellular defense mechanism against the toxicity of ozone.

METHODS

Animal exposure

Seven-week-old, male S.P.F. Wistar rats (145 ± 20 g) were exposed for 4 days to 0 or 1.5 ± 0.1 mg ozone/m^3 (0.75 ppm) in a 0.2-m^3 stainless steel

and glass inhalation chamber in which an air flow of 6 m^3/h was maintained. The air was filtered by a HEPA and an active carbon filter and was conditioned at a temperature of 22 ± 1°C and a relative humidity of 50 ± 5%. Ozone was generated in a silent discharge type ozone generator (Model 501, Fisher, Meckenheim, F.R.G.) in which oxygen is partially converted to ozone. The ozone-oxygen mixture, about 10 ml/min, was added to the inlet stream of the exposure chamber. The ozone concentration was measured continuously with an ozone analyser (Model 8810, Monitor Labs, San Diego, CA, USA). Calibration was performed with a primary standard before and after the exposure period. Daily calibration was performed using the analyser's internal ozone source. To maintain the ozone concentration at the desired value, a feedback control system was used, which controlled the generator oxygen flow by regulating a mass flow controller (Model F201, HiTec, Vorden, The Netherlands). A microprocessor (PDP 11/023, Digital Equipment Corporation, Maynard, U.S.A.), equiped with analog to digital and digital to analog convertors, adjusted the setpoint of the oxygen mass flow controller at regular intervals (5 min) proportional to the deviation of the measured ozone concentration from the desired value.

Isolation of cell cultures

Alveolar macrophages were isolated as described by Mason et al. [6] by the lavage of isolated perfused lungs from 6 ozone-exposed and 6 control rats. Approximately 10^6 cells were plated onto a 20-cm^2 culture dish (Costar, Cambridge, MA, U.S.A.) or — in case of in vitro ozone exposure — onto a plastic film dish with a 25-μm teflon bottom and a growth area of 18 cm^2 (Petriperm, Heraeus, Hanau, F.R.G). Alveolar macrophages were cultured in Ham's F10 medium (Flow, Irvine, Scotland), supplemented with $NaHCO_3$ (1.2 g/l), 10% newborn calf serum (Gibco, Glasgow, Scotland), 50IU/l of penicillin (Gist-Brocades, Delft, The Netherlands), and 50 mg/l streptomycin (Specia, Paris, France) at 37°C in a humid atmosphere containing 5% CO_2 in air. After 3 days, cells were exposed to ozone or harvested for biochemical determinations by scraping them from the culture dishes. Type II cells were also isolated from the lungs of 6 ozone-exposed and 6 control rats, following the method described by Mason et al. [6] using Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) instead of albumin for the density gradient centrifugation. Approximately 2×10^6 cells were cultured in a dish in Dulbecco's MEM, supplemented with $NaHCO_3$ (3.70 g/l) and 10% fetal calf serum (Gibco, Glasgow, Scotland) and penicillin and streptomycin at the same concentrations as described above. Cells were cultured at 37°C in a humid atmosphere containing 10% CO_2 in air. After 3 days cells were either exposed to ozone or dislodged with a solution containing 0.25% trypsin (Difco, Detroit, MI, U.S.A.) and 0.05% EDTA (Merck, Darmstadt, F.R.G.) and used for biochemical assays. The percentages of alveolar macrophages or type II cells in these primary cell cultures were determined by phagocytosis (carried out as described below) or by staining with Phosphine R (ICN, NY, U.S.A.), as described by Mason et al. [6].

Preparation of lung cytosol and cell samples

Cell samples were disrupted by freezing in liquid nitrogen and thawing at 37°C which was repeated twice. Perfused lungs were homogenized in a glass-teflon homogenizer with a 7-fold volume of ice-cold sucrose-mannitol medium as described by Mustafa et al. [7]. The homogenate was centrifuged at 105 000 *g* for 60 min at 4°C. The supernatant fraction was quickly frozen in liquid nitrogen and stored at −90°C until biochemical assay.

Biochemical parameters

G6PDH was measured according to Mustafa et al. [7]. GR was measured as described by Racker [8]. GSHPx activity was measured according to Lawrence and Burk [9] in 50 mM imidazole buffer pH 7.0, with hydrogen peroxide as a substrate. In this way the selenium-dependent GSHPx is measured, which might have a higher affinity for peroxides (K_m = 1—10 μM) [10], than the non-selenium-dependent GSHPx (K_m = 0.5—2.5 mM) [11]. Enzyme activities were expressed in units defined as μmol NADPH formed or used per minute. Protein was determined by the method of Lowry et al. [12] with crystalline bovine serum albumin as the standard.

In vitro exposure to ozone

Before exposure, fresh medium without serum was added to the cells and the dishes were closed in such a way that ozone was unable to enter the dish except by diffusion through the teflon bottom. Cells were exposed to ozone as previously described [13]. The amount of ozone which diffused through the teflon film into the dish was measured using the indigo disulfonic acid method [14].

Parameters for cellular damage

The viability of either alveolar macrophages or type II cells, was assessed by counting the percentage of trypan blue excluding cells among the cells attached to the plastic film, 5—10 min after addition of trypan blue (BDH chemicals, Poole, U.K.) (0.5% final conc.). For alveolar macrophages a functional parameter was included as well: the percentage of phagocytosing cells was determined after incubating the cells for 1.5 h at 37°C in the presence of approximately 10^7 dead yeast cells, coloured by boiling them for 30 min in a congo red (Fluka, Buchs, Switzerland) PBS solution.

Statistical analysis of data

Data are presented as mean ± standard error of the mean, and statistical analysis was carried out using the paired Student's *t*-test, unless indicated otherwise.

RESULTS

Lungs of rats exposed to 1.5 ± 0.1 mg ozone/m^3 for 4 days showed a marked increase in the activities of G6PDH ($P < 0.001$), GR ($P < 0.001$) and

GSHPx ($P < 0.001$), when expressed per lung (Table I). Because lung weight ($P < 0.01$) and cytosolic lung protein ($P < 0.001$) also increased after exposure to ozone (Table I), increases in enzyme activities were in fact less pronounced or even disappeared when expressed per gram lung or per gram of cytosolic lung protein (Table I).

Alveolar macrophages isolated from control rats showed no difference in the percentage of phagocytosing cells compared to cells isolated from ozone-exposed rats. These percentages amounted to 96 ± 1% and to 94 ± 1% for alveolar macrophages from control and ozone-exposed rats, respectively. Type II cell cultures from control or ozone-exposed rats contained the same percentage of type II cells, 66 ± 5% and 61 ± 6%, respectively, as determined by staining with phosphine R. The remaining percentages consisted mainly of fibroblasts. Furthermore, cell cultures isolated from control rat lungs did not differ from those from ozone-exposed rat lungs with respect to their light microscopical appearance (data not shown).

From Table II it can be seen that alveolar macrophages as well as type II

TABLE I

EFFECTS OF OZONE EXPOSURE (1.5 ± 0.1 mg OZONE/m³; 4 DAYS) ON (RELATIVE) LUNG WEIGHT, ON PROTEIN CONTENT AND ON G6PDH, GR AND GSHPx ACTIVITIES IN RAT LUNG CYTOSOL.

Parameter	Control	Ozone-exposed
Animal weight (g)	149 ± 7	145 ± 9
Lung weight (g)	0.91 ± 0.04	1.33 ± 0.06***
% of animal weight	0.62 ± 0.03	0.94 ± 0.07**
Cytosolic protein		
mg/lung	23.3 ± 0.8	35.5 ± 1.1***
mg/g lung	25.7 ± 1.6	27.1 ± 1.7
G6PDH		
U/lung	3.32 ± 0.11	6.86 ± 0.44***
U/g lung	3.66 ± 0.18	5.23 ± 0.40**
U/g protein	143 ± 3	193 ± 9***
GR		
U/lung	1.15 ± 0.04	1.76 ± 0.07***
U/g lung	1.26 ± 0.06	1.34 ± 0.09
U/g protein	49 ± 1	50 ± 2
GSHPx		
U/lung	3.70 ± 0.20	7.04 ± 0.41***
U/g lung	4.09 ± 0.27	5.36 ± 0.39*
U/g protein	159 ± 5	198 ± 7***

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (Unpaired student's t-test).
Units (U) are defined as μmol NADPH formed or used per minute. Values are the mean ± S.E.M. of 6 animals.

TABLE II

ACTIVITIES OF G6PDH, GR AND GSHPx IN ALVEOLAR MACROPHAGES (AM) OR TYPE II CELLS (tII) ISOLATED FROM CONTROL OR OZONE-EXPOSED RATS (1.5 ± 0.1 mg OZONE/m³; 4 DAYS)

Parameter	Cell type	Activity	Control	Ozone-exposed
G6PDH	AM	U/10⁶ cells	2.25 ± 0.40	3.01 ± 0.31*
		U/g protein	239 ± 56	281 ± 77
	tII	U/10⁶ cells	3.82 ± 0.62	5.14 ± 0.68
		U/g protein	127 ± 15	143 ± 11
GR	AM	U/10⁶ cells	1.52 ± 0.08	1.31 ± 0.10
		U/g protein	70 ± 6	76 ± 7
	tII	U/10⁶ cells	1.49 ± 0.10	1.62 ± 0.14
		U/g protein	38 ± 1	38 ± 1
GSHPx	AM	U/10⁶ cells	5.23 ± 1.07	8.22 ± 2.04
		U/g protein	471 ± 213	818 ± 142*
	tII	U/10⁶ cells	0.89 ± 0.20	2.13 ± 0.51**
		U/g protein	38 ± 10	84 ± 14**

*$P < 0.05$, **$P < 0.01$.
Units (U) are defined as μmol NADPH formed or used per minute. Values are the mean ± S.E.M. of 5 experiments.

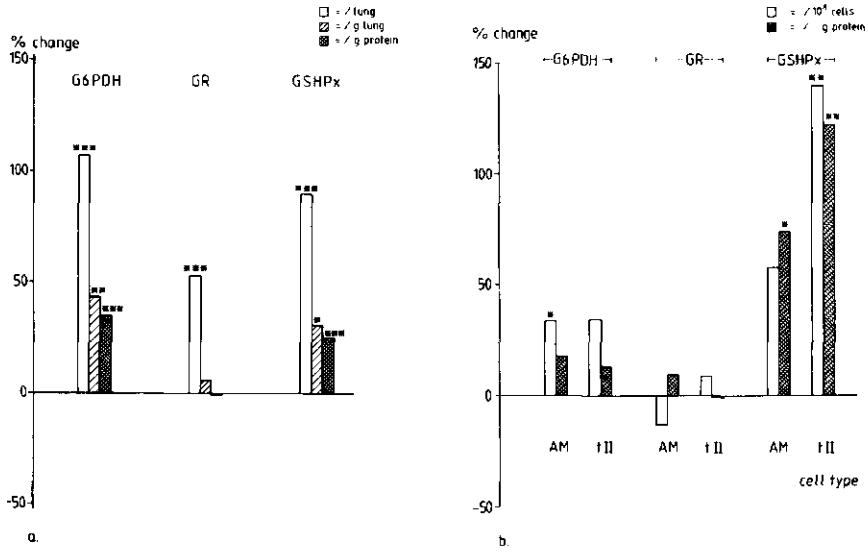

Fig. 1. Biochemical changes in (a) rat lung cytosol and (b) isolated alveolar macrophages (AM) and type II cells (tII), caused by exposure of the rats to ozone (1.5 ± 0.1 mg ozone/m³; 4 days) and presented as percentages increase or decrease with regard to control values. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

TABLE III

EFFECT OF IN VITRO EXPOSURE TO OZONE ON ALVEOLAR MACROPHAGES (AM) (n = 6) AND TYPE II CELLS (tII) (n = 3) ISOLATED FROM CONTROL AND OZONE-EXPOSED RATS (1.5 ± 0.1 mg OZONE/m^3; 4 DAYS)

Trypan blue exclusion and phagocytosis are presented as percentages of unexposed control values, (n.s. = not significant).

Cell type	Culture from control or ozone-exposed rat	Ozone dose in vitro in μg ozone/dish	Trypan blue exclusion % of control	Phagocytosis % of control
AM	Control	0.47 ± 0.14	62.1 ± 5.3	41.0 ± 10.9
	Ozone-exposed	0.47 ± 0.14	62.1 ± 4.9 n.s.	45.5 ± 10.5 n.s.
tII	Control	0.19 ± 0.09	48.9 ± 4.5	—
	Ozone-exposed	0.19 ± 0.09	51.7 ± 5.3 n.s.	—

cells from ozone-exposed rats differed from cells derived from control rats with respect to biochemical parameters. Whereas the GR activity showed no significant changes, the activity of GSHPx showed a significant increase in both alveolar macrophages and type II cells from ozone-exposed rats and the G6PDH activity showed a tendency to be increased in cells from ozone-exposed rats.

In Figs. 1a and b biochemical changes in rat lung cytosol and in the isolated cell cultures caused by exposure of the rats to ozone, are presented as percentages increase or decrease with regard to control values. From the results presented in Table III it can be seen, that neither alveolar macrophages nor type II cells from ozone-exposed rats showed an increased resistance towards an in vitro ozone exposure, compared to their respective control cells isolated from unexposed rats.

DISCUSSION

In the present study it was demonstrated that lungs of ozone-exposed rats contained increased activities of G6PDH, GR and GSHPx, which is in accordance with several other studies [4,5,15,16]. Since these increases were less pronounced or disappeared, when expressed per gram lung, or per gram of cytosolic protein, it might be concluded that in this study at least part of the increases in enzyme activities in the lung were caused by cell proliferation.

The enzyme activities of isolated control cells, expressed per 10^8 cells or per gram of protein were generally higher in alveolar macrophages than in type II cells. Only the G6PDH activity expressed per 10^8 cells was higher in type II cells compared to alveolar macrophages, which is in accordance with results from histochemical assays reported in literature [17].

Alveolar macrophages and type II cells isolated from ozone-exposed rats showed some biochemical differences compared to cells from unexposed rats, and these differences might be compared to the effects measured in cytosol from whole rat lung homogenates. Comparisons can best be made between activities expressed in the same unit, i.e. per gram of protein. G6PDH activity was increased in lungs from rats exposed to ozone ($P < 0.001$) and showed the same tendency in isolated alveolar macrophages and type II cells. GR activity did not change significantly in whole lungs and neither in alveolar macrophages or type II cells. GSHPx activity was increased in whole lung homogenates ($P < 0.001$), in alveolar macrophages ($P < 0.05$) and in type II cells ($P < 0.01$) from ozone-exposed rats. So the effects measured in whole lung homogenates ran parallel with metabolic changes detected in isolated cells. With respect to the GSHPx activity, a marked difference appeared in the extent of the induction of the GSHPx activity in the whole lung compared to the increase in GSHPx activity measured in isolated lung cells. Whereas the GSHPx activity expressed per gram of cytosolic lung protein was increased by 35% ($P < 0.001$) in the lungs of rats exposed to ozone, the increase in GSHPx activity was far more pronounced in both alveolar macrophages (+74%, $P < 0.05$) and type II cells (+122%, $P < 0.01$), isolated from the lungs of ozone exposed rats.

Increases in G6PDH and GSHPx activities expressed per gram cytosolic protein in both rat lung cytosol and cell cultures isolated from ozone-exposed rats, might be caused by: (i) activation of silent enzyme sources; and/or (ii) a de novo synthesis, both of either enzymes with an increased specific activity or of enzymes with the same specific activity as those in control rat lungs. From the data presented none of these mechanisms for increases of enzyme activities can be excluded.

Furthermore, it was demonstrated in this study, using an in vitro ozone exposure system, that neither alveolar macrophages nor type II cells from ozone-exposed rats showed an increase in their resistance towards ozone, as measured by trypan blue exclusion and phagocytosis. This, in spite of the pronounced increase in the GSHPx activity of these cells. These results imply that an increased GSHPx activity is not related to an increased cellular resistance to ozone.

The hypothesis that GSHPx catalysed reduction of toxic fatty acid hydroperoxides, formed as a consequence of ozone intoxication, may play a major role in protecting lungs against ozone-induced injury was also contradicted by other reports in the literature. McCay et al. [18] reported that the GSHPx system was not able to reduce lipid hydroperoxides, present in phospholipid emulsions, to the corresponding alcohols. Chow et al. [19] reported that the relative activities of GSHPx in the lungs of rats and monkeys, expressed per mg of protein, were unrelated to the susceptibility of these animals to oxidant damage. In addition, another study reported by Chow [16] showed that even though rat lung GSHPx activity had completely returned to its control level 9 days after an initial ozone exposure, the rats still showed a reduced mortality rate when subjected to a subsequent

exposure. Furthermore, it is known that younger rats are more resistant towards ozone [1,20] in spite of relatively less efficient pulmonary antioxidant enzymes [21]. Nambu and Yokoyama [18] demonstrated that ozone tolerance in rats occurred 1 day before the enhancement of the GSHPx activity. Elsayed et al. [22] reported that mice fed a selenium-rich diet, in spite of a 3-fold increase in their lung GSHPx activity, did not show a significant alteration in their resistance or their pulmonary sensitivity to ozone, compared to dietary selenium-deficient mice. Finally, the results presented in this study are in accordance with recent data [23], which show that the protective mechanism of glutathione in lung cells exposed to ozone, is not mediated through the action of GSHPx; A549 cells, which do not contain a detectable amount of GSHPx, did show an increased sensitivity to ozone, when they were artificially depleted of GSH.

Although it is clearly demonstrated by the present results that the activity of GSHPx might be induced on a cellular level after exposure of rats to ozone, it is also shown that this increased enzyme activity does not represent a key mechanism for increased resistance of cells in vitro exposed to ozone.

From the results presented, however, it can not be concluded that the increased enzyme activity is merely a non-specific artificial response of these lung cells to exposure to ozone, because it can not be excluded that some specific biochemical cellular process, which is not of prime importance for the survival of individual cells, but which might be important for the overall resistance of the whole lung (*p/e* synthesis of surfactant), benefits by the increase in GSHPx activity.

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In preparation*

CHAPTER 6b

GLUTATHIONE PATHWAY ENZYME ACTIVITIES AND THE SENSITIVITY TOWARDS NITROGEN DIOXIDE OF LUNG CELLS ISOLATED FROM NITROGEN DIOXIDE EXPOSED RATS

I.M.C.M. RIETJENS

SUMMARY

After exposure of rats to 20 ± 1 mg nitrogen dioxide/m^3 (10.6 ppm) for 4 days, the activities of glucose-6-P dehydrogenase (G6PDH), glutathione reductase (GR) and glutathione peroxidase (GSHPx) were measured in the cytosolic lung fraction, in isolated alveolar macrophages and in type II cells from exposed and control rats.

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*Data presented in this section will be part of a manuscript entitled: ALTERATIONS IN RAT LUNG AND ISOLATED LUNG CELLS IN RESPONSE TO IN VIVO AND IN VITRO NITROGEN DIOXIDE EXPOSURE. A BIOCHEMICAL, ENZYM HISTOCHEMICAL AND MORPHOLOGICAL STUDY.

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Enzyme activities in whole lung homogenates expressed per gram of protein showed the following nitrogen dioxide induced changes. G6PDH activity was increased ($p < 0.001$), GR was increased ($p < 0.001$) but GSHPx activity showed no change. The increase in G6PDH activity was comparable to data previously found with ozone. Based on the oxidant concentrations applied in these studies, ozone was 14 times more effective than nitrogen dioxide with regard to G6PDH induction.

The effect of nitrogen dioxide on GR and GSHPx activity appeared to be different from that of ozone. In contrast to nitrogen dioxide, ozone induced GSHPx activity, but did not affect GR activity, whereas nitrogen dioxide induced GR activity and did not affect GSHPx activity. These results point to a different mechanism of action of ozone and nitrogen dioxide in vivo.

Alveolar macrophages and type II cells isolated from the lungs of rats exposed to nitrogen dioxide showed no significant change in GR, but increased G6PDH and GSHPx activities.

From this it follows that nitrogen dioxide exposure induces biochemical changes on a cellular level.

Furthermore it was demonstrated, using an in vitro system in which the cells could be exposed to nitrogen dioxide, that neither alveolar macrophages nor type II cells derived from nitrogen dioxide exposed rats showed an increased resistance versus nitrogen dioxide, as compared to cells from unexposed rats.

These results clearly indicate that the nitrogen dioxide induced elevation of cellular GSHPx activity is not related to an increased cellular resistance towards the oxidative compound.

INTRODUCTION

Nitrogen dioxide is a well known constituent of polluted industrial and urban atmospheres. Its toxicity to human and animal lung has been described (1).

In addition to morphologic and physiologic effects, nitrogen dioxide is known to cause biochemical changes in the lungs of exposed animals. After exposure to 2-10 ppm nitrogen dioxide for several days, the activities of the enzymes of the glutathione pathway, glucose-6-P-dehydrogenase (G6PDH), glutathione reductase (GR) and glutathione peroxidase (GSHPx), were demonstrated to be increased (2;3;4).
It has been suggested that this phenomenon reflects a mechanism for cellular protection against nitrogen dioxide, as the enzymes may detoxify lipid hydroperoxides thus preventing further oxidative damage (2;4).
However, it is not known whether the nitrogen dioxide induced increases in glutathione pathway enzyme activities are caused by intrinsic cellular metabolic changes and/or by migration, proliferation or replacement of certain cell types in the lung, known to occur following nitrogen dioxide exposure (5). Furthermore it may be questioned whether increases in G6PDH, GR and GSHPx, measured in whole lung homogenates, can be coupled to an increased cellular resistance to nitrogen dioxide.

Recently published observations with alveolar macrophages and type II cells isolated from the lungs of ozone exposed and control animals, demonstrated that the increases in glutathione pathway enzyme activities measured in whole lung homogenates really reflect changes occurring on a cellular basis (6). But it also appeared that pronounced increases in cellular GSHPx activity did not result in an increased cellular resistance towards ozone in vitro. This indicated that the phenomena of GSHPx induction and ozone tolerance may not be related (6).
Other data published recently (7) clearly demonstrated that the mechanism of action of ozone and nitrogen dioxide towards in vitro exposed alveolar macrophages proceed by different reactive intermediates and thus by different reaction pathways. Therefore it is worthwhile to investigate the effect of nitrogen dioxide on G6PDH, GR

and GSHPx activities in cells isolated from the lungs of control and nitrogen dioxide exposed rats and to determine their sensitivity towards an *in vitro* exposure to nitrogen dioxide. This in analogy to earlier experiments with ozone, described recently.

Such a study with nitrogen dioxide may provide insight into the questions i) whether - as for ozone - increases in glutathione pathway enzyme activities induced by nitrogen dioxide in whole lung reflect inductions on a cellular level and ii) if so, whether increased cellular activities of glutathione pathway enzymes result in an increased cellular resistance versus nitrogen dioxide.

In addition, comparison of the results to those published recently for ozone, may provide insight into iii) (dis)similarities between ozone and nitrogen dioxide induced effects *in vivo*.

METHODS

Animal exposure

9-Week old male S.P.F. Wistar rats (200 $\pm$ 20 g) were exposed for 4 days to 0 or 20 $\pm$ 1 mg nitrogen dioxide/m^3 (10.6 ppm) in a 0.2 m^3 stainless steel and glass inhalation chamber, according to the method of Rombout et al. (8).

Isolation of cell cultures

Alveolar macrophages and type II cells were isolated and cultured as described previously (7). Cells isolated from the lungs of 6 control or 6 nitrogen dioxide exposed rats were pooled to give two independent samples. The mean value of these two cell samples, each derived from 3 rats, provided one individual experimental data for each parameter determined.

The percentages of alveolar macrophages or type II cells in the primary cultures were determined by phagocytosis (carried out as described below) or by staining with phosphine R (ICN, NY USA) as described by Mason et al. (9), respectively.

Biochemical parameters

Preparation of lung cytosol and cell samples was carried out as described in a previous report (7). G6PDH, GR and GSHPx activity as well as protein content, were all determined as previously described (7).

In vitro exposure to nitrogen dioxide

Before exposure, fresh medium without serum was added to the cells and the dishes were closed in such a way that nitrogen dioxide was unable to enter the petriperm dish (Petriperm, Heraeus, Hanau, West Germany), except by diffusion through the teflon film to which the cells were attached. Thus cells were exposed to nitrogen dioxide by means of gas diffusion through the teflon bottom of the dish. This was achieved by placing the culture dishes in a 36 litre perspex fumigation box at 37°C (10). The inlet gas mixture contained 85% N_2, 5% CO_2, 10% O_2 and an experimental amount of nitrogen dioxide. Nitrogen dioxide (0.9% NO_2 in N_2) was purchased from Matheson (Oevel, Belgium). The amount of nitrogen dioxide which diffused through the teflon film into the dish was measured using the method described by Huygen (11) and a nitrite equivalent of nitrogen dioxide, i.e. a Saltzman factor of 0.67.

Parameters for cellular damage

The viability of alveolar macrophages and type II cells was determined by trypan blue exclusion. For alveolar macrophages a functional parameter, viz. phagocytosis, was included as well.
Trypan blue exclusion and phagocytosis were determined as previously described (7).

Statistical analysis of data

Data are presented as mean $\pm$ standard error of the mean, and statistical analysis was carried out using the paired Student's t-test, unless indicated otherwise.

RESULTS

Table I and Figure 1a demonstrate that lungs of rats exposed for 4 days to 20 $\pm$ 1 mg nitrogen dioxide/m^3 (10.6 ppm) contain significantly ($p < 0.001$) increased levels of G6PDH, GR and GSHPx activities per whole lung. Because both lung weight ($p < 0.001$) and cytosolic lung protein ($p < 0.001$) also increased significantly after nitrogen dioxide exposure (Table I), increases in enzyme activities were less pronounced - although still significant - when expressed per gram lung or per gram of cytosolic lung protein. Only GSHPx did not show any change when expressed per gram of cytosolic protein.

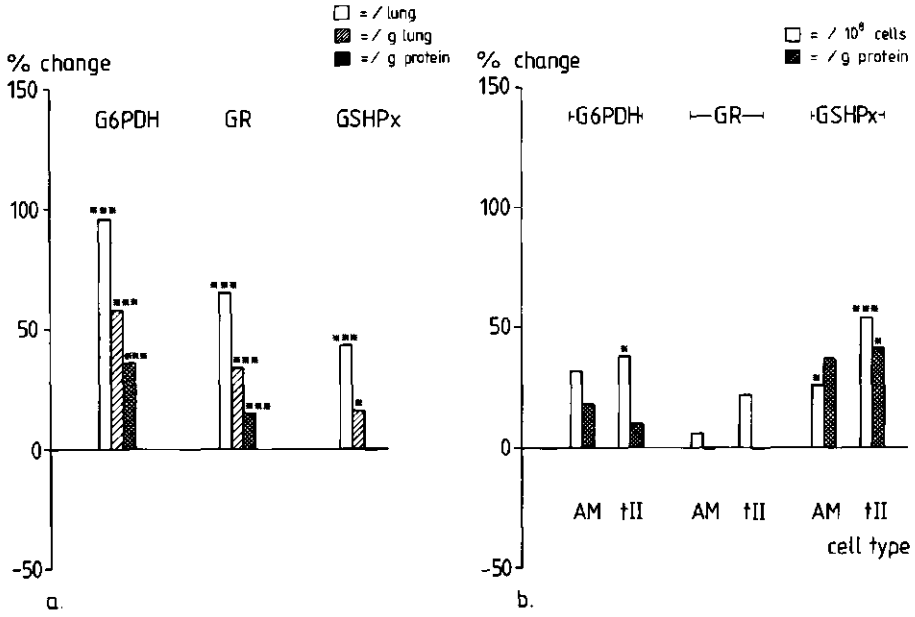

Figure 1. Biochemical changes in a) rat lung cytosol and b) isolated alveolar macrophages (AM) and type II cells (tII), caused by exposure of the rats to nitrogen dioxide (20 $\pm$ 1 mg nitrogen dioxide/m^3; 4 days) and presented as percentages increase with regard to control values, * $p < 0.05$, *** $p < 0.001$.

TABLE I

EFFECTS OF NITROGEN DIOXIDE EXPOSURE (20 ± 1 mg NITROGEN DIOXIDE/m^3; 4 DAYS) ON (RELATIVE) LUNG WEIGHT, ON PROTEIN CONTENT AND ON G6PDH, GR AND GSHPx ACTIVITIES IN RAT LUNG CYTOSOL.

Parameter	Control	Nitrogen dioxide exposed	
Animal weight (g)	220 ± 7	203 ± 5	
Lung weight (g)	0.97 ± 0.03	1.20 ± 0.02	***
% of animal weight	0.44 ± 0.01	0.59 ± 0.01	***
Cytosolic protein			
mg/lung	24.1 ± 0.6	34.8 ± 1.1	***
mg/g lung	24.9 ± 0.2	29.0 ± 0.7	***
G6PDH			
U/lung	4.38 ± 0.07	8.57 ± 0.37	***
U/g lung	4.54 ± 0.09	7.16 ± 0.27	***
U/g protein	182 ± 3	247 ± 7	***
GR			
U/lung	1.29 ± 0.04	2.13 ± 0.07	***
U/g lung	1.33 ± 0.01	1.78 ± 0.06	***
U/g protein	53 ± 1	61 ± 2	***
GSHPx			
U/lung	2.92 ± 0.11	4.19 ± 0.24	***
U/g lung	3.02 ± 0.10	3.49 ± 0.17	*
U/g protein	121 ± 9	120 ± 9	

* $p < 0.05$, *** $p < 0.001$ (unpaired Student's t-test).
Units are defined as μmol NADPH formed or used per minute. Values are the mean ± S.E.M. of 6 animals.

Table II summarizes some of the characteristics of the populations of alveolar macrophages and type II cells, isolated from the lungs of control or nitrogen dioxide exposed rats. It follows from these results that a markedly increased number of alveolar macrophages could be isolated from the lungs of exposed animals ($p < 0.001$). Cultures of

alveolar macrophages derived from these populations showed the same percentage of phagocytosing cells (Table II).

Type II cell cultures derived from control and nitrogen dioxide exposed rats contained the same percentage of type II cells, as determined by staining with phosphine R (Table II).

From light microscopical appearance of these type II cell cultures it followed that the remaining percentages consisted mainly of fibroblasts (data not shown).

TABLE II

CHARACTERISTICS OF POPULATIONS OF ALVEOLAR MACROPHAGES (AM) AND TYPE II CELLS (tII) ISOLATED FROM THE LUNGS OF CONTROL AND NITROGEN DIOXIDE EXPOSED RATS (20 ± 1 mg NITROGEN DIOXIDE/m^3; 4 DAYS).

Parameter	Cell type	Control	Nitrogen dioxide exposed
- number of isolated cells 10^6 cells/rat	AM	5.64 ± 0.18	12.28 ± 1.08 **
	tII	9.27 ± 0.47	10.55 ± 1.13
- percentage of specific cells in culture at t = 3 days[1)]	AM	97.5 ± 0.4	97.0 ± 0.7
	tII	67.5 ± 1.5	66.1 ± 2.2

[1)] AM: determined by phagocytosis, tII by staining with phosphine R.

** $p < 0.01$.

Table III and Figure 1b show differences in biochemical characteristics between alveolar macrophages as well as type II cells isolated from control or nitrogen dioxide exposed rats. G6PDH activity showed a tendency to be increased in cells from nitrogen dioxide exposed cells. GR activity showed no significant changes. And GSHPx activity showed a significant increase in both alveolar macrophages ($p < 0.05$) and type II cells ($p < 0.001$) when expressed per 10^8 cells.

These increases in GSHPx activity became less pronounced when expressed per gram of protein, although the difference was still significant ($p < 0.05$) for alveolar type II cells from nitrogen dioxide exposed rats.

TABLE III

ACTIVITIES OF G6PDH, GR AND GSHPx IN ALVEOLAR MACROPHAGES (AM) OR TYPE II CELLS (tII) ISOLATED FROM CONTROL OR NITROGEN DIOXIDE EXPOSED RATS (20 ± 1 mg NITROGEN DIOXIDE/m^3; 4 DAYS).

Parameter	Cell type	Activity	Control	Nitrogen dioxide exposed
G6PDH	AM	U/10^8 cells	2.53 ± 0.29	3.35 ± 0.33
		U/g protein	149 ± 19	176 ± 14
	tII	U/10^8 cells	3.16 ± 0.26	4.35 ± 0.24 *
		U/g protein	96 ± 7	106 ± 8
GR	AM	U/10^8 cells	1.27 ± 0.05	1.35 ± 0.08
		U/g protein	134 ± 19	134 ± 15
	tII	U/10^8 cells	1.66 ± 0.24	2.03 ± 0.12
		U/g protein	53 ± 5	53 ± 3
GSHPx	AM	U/10^8 cells	3.17 ± 0.11	3.98 ± 0.12 *
		U/g protein	367 ± 88	501 ± 87
	tII	U/10^8 cells	0.52 ± 0.04	0.80 ± 0.12 ***
		U/g protein	22 ± 2	31 ± 3 *

* $p < 0.05$, *** $p < 0.001$.

Units (U) are defined as µmol NADPH formed or used per minute. Values are the mean ± S.E.M. of 5 experiments.

Finally the results presented in Table IV show that neither alveolar macrophages nor type II cells from nitrogen dioxide exposed rats demonstrated an increased resistance towards an *in vitro* exposure to nitrogen dioxide, compared to their respective control cells derived from unexposed rats.

TABLE IV

EFFECT OF *IN VITRO* EXPOSURE TO NITROGEN DIOXIDE ON ALVEOLAR MACROPHAGES (AM) (n = 5) AND TYPE II CELLS (tII) (n = 5) ISOLATED FROM CONTROL AND NITROGEN DIOXIDE EXPOSED RATS (20 ± 1 mg NITROGEN DIOXIDE/m^3; 4 DAYS).

Trypan blue exclusion and phagocytosis are presented as percentages of unexposed control values; n.s. = not significant.

Cell type	Culture from control or nitrogen dioxide exposed rat	Nitrogen dioxide dose *in vitro* in µg nitrogen dioxide/dish	Trypan blue exclusion % of control	Phagocytosis % of control
AM	control	3.88 ± 0.11	69.1 ± 4.2	54.3 ± 3.3
	nitrogen dioxide exposed	3.88 ± 0.11	70.2 ± 4.8 n.s.	59.9 ± 6.0 n.s.
tII	control	2.23 ± 0.14	56.6 ± 10.6	-
	nitrogen dioxide exposed	2.23 ± 0.14	63.7 ± 13.9 n.s.	-

DISCUSSION

Lung homogenates from nitrogen dioxide exposed rats contained increased activities of G6PDH, GR and GSHPx, a phenomenon reported before by Chow et al. (2) and Sagai et al. (3;4). From the observation presented in this study, that these increases were less pronounced

when expressed per gram lung or per gram of cytosolic protein it follows that at least part of the increases in lung enzyme activities were caused by cell proliferation. This also follows from the observation that the number of alveolar macrophages isolated from the lungs of nitrogen dioxide exposed rats was significantly greater (+ 118%, $p < 0.01$) than the number of alveolar macrophages isolated from control rats.

The increase in GSHPx completely disappeared when expressed per gram of cytosolic protein. This observation differs from data presented by Sagai et al. (3;4) and Mochitate et al. (12) who reported a 10 to 20% increase in GSHPx specific activity in the lungs of rats exposed for 4 days to 10 or 4 ppm nitrogen dioxide respectively. However, the data are in accordance with results reported by Chow et al. (2) who concluded that in the lung cytosol of rats exposed to 6.2 ppm nitrogen dioxide for 4 days the activities of G6PDH and GR, but not of GSHPx were significantly increased over those of unexposed control samples.

Previously published data (7) demonstrated that lungs of ozone exposed rats contained increased activities of G6PDH, GR and GSHPx as well, an observation in accordance with several other studies in the literature (2;13). Comparison of the changes induced by nitrogen dioxide in this study on one hand, and by ozone in our previous study, reveals a comparable effect on G6PDH activity.

This similar biochemical effect is achieved by 4 days exposure to 0.75 ppm ozone (7) or 10.6 ppm nitrogen dioxide, indicating that with respect to G6PDH induction ozone is about 14 times more effective than nitrogen dioxide. This difference in reactivity is in accordance with several other *in vivo* studies presented in the literature, which reported ozone to be 10 to 25 times more toxic than nitrogen dioxide (2; 14; 15; 16).

However, the effect of nitrogen dioxide on GR and GSHPx activity was different from that of ozone, especially when expressed per gram of cytosolic protein. This appears from the observation that GR activity

was significantly enhanced ($p < 0.001$) and GSHPx activity showed no increase upon exposure to nitrogen dioxide whereas ozone exposure was reported to result in an unchanged GR activity but a significantly ($p < 0.001$) increased activity of GSHPx per gram of cytosolic protein. These results point to a different mechanism of action of nitrogen dioxide and ozone in vivo.

Alveolar macrophages and type II cells isolated from the lungs of rats exposed to nitrogen dioxide showed no significant change in GR activity, but increased G6PDH and GSHPx activities. From this it is concluded that nitrogen dioxide exposure of rats induces biochemical changes on a cellular level.

Comparison of the biochemical changes in whole lung cytosol on one hand and isolated lung cells on the other can be made between activities expressed in the same unit, viz. per gram of protein. G6PDH activity was increased in lung homogenates from rats exposed to nitrogen dioxide ($p < 0.001$) and showed the same tendency in isolated alveolar macrophages and type II cells. Discrepancies occur with respect to GR activity - induced in whole lung homogenates but not on a cellular basis - and with regard to GSHPx activity - increased on a cellular basis but not per gram of cytosolic whole lung protein -. These discrepancies must probably be ascribed to unknown, additional changes in lung cell populations of nitrogen dioxide exposed rats.

In addition it appeared, using an in vitro nitrogen dioxide exposure system, that neither alveolar macrophages nor type II cells derived from nitrogen dioxide exposed rats showed an increase in their resistance towards nitrogen dioxide. This in spite of their significantly increased GSHPx activity.

These results clearly indicate that the nitrogen dioxide induced elevation of cellular GSHPx activity is not related to an increased cellular resistance versus the oxidative compound. The same conclusion has recently been drawn for the ozone induced elevation of cellular

GSHPx activity which was even far more pronounced than the induction evoked by nitrogen dioxide (7). However, as also stated before (7), it can not be concluded that the increased enzyme activity is merely a non-specific response of these lung cells to nitrogen dioxide. This because it can not be excluded that some specific cellular function which is not of prime importance for the survival of individual cells, but which might be of importance for the resistance of the whole lung (p/e surfactant synthesis), benefits by the increased glutathione pathway enzyme activities.

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CHAPTER 7

THE ROLE OF GLUTATHIONE AND CHANGES IN THIOL HOMEOSTASIS IN CULTURED LUNG CELLS EXPOSED TO OZONE

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SUMMARY

Cells of an alveolar type II cell-line (A549) were exposed to ozone, using an in vitro exposure model. In this study, attention was focussed on the cellular glutathione system. It was demonstrated that cellular levels of both reduced (GSH) and oxidized glutathione (GSSG) were significantly reduced after exposure of the cells to ozone. When A549 cells were incubated with methionine sulfoximine and diethylmaleate, glutathione levels were depleted, and the cells showed a marked increase in sensitivity towards ozone. Some of the possible mechanisms by which the observed effects might be explained were investigated. It was shown that glutathione lost from the cells was not incorporated into "mixed disulfides", but could be detected in the surrounding medium. Furthermore, it was shown that A549 cells do not contain any detectable glutathione peroxidase activity. Therefore it was concluded that glutathione peroxidase-catalysed reduction of lipid peroxides could not be responsible for the observed protective role of glutathione. Finally some other mechanisms by which glutathione might accomplish its antioxidant effect are discussed.

Keywords: Ozone; Glutathione; "Mixed disulfides"; Glutathione peroxidase; In vitro

INTRODUCTION

Glutathione is one of the cellular reducing substances and might play, as

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Abbreviations: DEM, diethylmaleate; GSH, reduced glutathione; GSSG, oxidized glutathione; MSO, methionine sulfoximine; OPT, *o*-phtalaldehyde.

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such, an important role in the defense mechanism of cells against damage by the oxidative air pollutant ozone.

In vivo it was demonstrated that the glutathione level of erythrocytes from mice [1] and men [2] exposed to ozone, was reduced. In addition it was reported that the non-protein sulfhydryl level (representing mainly glutathione) of rat lung [3] and the glutathione level of mouse lung [4] decreased after exposure of the animals to ozone.

Based on short-term in vivo experiments a mechanism was suggested in which glutathione might function in the enzymatic detoxification of fatty acid hydroperoxides formed as a result of ozone-induced lipid peroxidation [5]. This hypothesis is based primarily on the observation that the activities of GSH peroxidase, GSSG reductase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat lungs increased above their respective controls after several days of exposure of the rats to ozone.

Furthermore, it was shown that during the exposure of rats to relatively high concentrations of ozone, glutathione became bound to lung tissue protein sulfhydryl groups, which resulted in the formation of "mixed disulfides" [6]. Thus glutathione might protect cellular sulfhydryl groups from irreversible oxidation by ozone or its oxidative initial product(s).

In addition in vitro studies by Menzel [7] showed that GSH in solution was oxidized as a consequence of exposure to ozone, indicating that glutathione might possibly protect cells from ozone intoxication by scavenging the oxidative compound(s).

In this study, the possible role of glutathione in the defense mechanism of cells against ozone damage was investigated using an in vitro system in which cell cultures can be exposed to ozone [8].

An advantage of the cell model is that biochemical changes of homogeneous cell populations can be studied, which eliminates one of the problems encountered by measurements using in vivo exposed lung tissue, namely, whether biochemical changes should be ascribed to real metabolic changes and/or to loss, proliferation or replacement of certain cell types in the lung with different biochemical characteristics [9].

METHODS

Cell culture

Cells of the alveolar type II cell-line A549 were cultured in Ham's F10 medium (Flow, Irvine, Scotland), supplemented with $NaHCO_3$ (1.2 g/l), 10% newborn calfserum (Gibco, Glasgow, Scotland), 50 IU/l of penicillin (Gist-Brocades, Delft, The Netherlands), and 50 mg/l streptomycin (Specia, Paris, France) in 75-cm^2 tissue culture flasks (Costar, Cambridge, MA, USA) at 37°C in a humid atmosphere containing 5% CO_2 in air. Before use, cells were dislodged with a 0.25% trypsin solution (Difco, Detroit, MI) and 0.05% EDTA (Merck, Darmstadt, F.R.G.).

For each experiment approximately 2×10^5 cells were plated onto a

plastic film dish (Petriperm, Heraeus, Hanau, F.R.G.) with a 25-μm teflon bottom and a growth area of 18 cm^2.

Cells were cultured for 3 days until a confluent monolayer was obtained.

Exposure to ozone

Before exposure, fresh medium without serum was added to the cells and the dishes were closed in such a way, that no ozone entered the dish except by diffusion through the teflon bottom.

Cells were exposed to ozone as previously described [8]. Briefly, culture dishes were placed in a 36 litre perspex fumigation box at 37°C. The inlet gas mixture contained 85% N_2, 5% CO_2, 10% O_2 and an experimental amount of ozone. The total exposure period, including the time needed for increase and decrease of the ozone level was 2.5 h. The ozone dose in the plastic film dishes was measured using the indigo disulfonic acid method [10].

Trypan blue exclusion

The viability of the cells was assessed by counting the number of trypan blue excluding cells out of 400, 5—10 min after addition of trypan blue (BDH Chemicals, Poole, England) (0.5% final concentration).

GSH and GSSG

Glutathione, both the reduced and the oxidized form, were measured by the method of Hissin and Hilf [11] with slight modifications. Cells were disrupted in phosphate-EDTA buffer by freezing (in liquid N_2) and thawing at 37°C, which was repeated twice. No HPO_3 was added , but omission of this protein precipitant did not influence the measurement of GSH or GSSG in these cell preparations.

Although the validity of this o-phtalaldehyde (OPT) method of Hissin and Hilf [11] for the estimation of GSH is generally accepted [12], its accuracy to determine GSSG in biological samples is still under discussion [13,14]. Dowex-1 formate chromatography of an A549 sample, carried out as described by Beutler and West [13], resulted in an elution pattern with only 25—30% of the OPT-reactive material washed through the column before application of the sodium formate gradient instead of the 80—85% reported by Beutler and West [13]. So disturbance of the OPT-GSSG measurement in these cell samples apparently is not as pronounced as described for the liver extract [13].

Although the method cannot be used to determine absolute cellular GSSG amounts, it can be used to detect whether chenges in GSH values are accompanied by concomitant changes in GSSG levels, because these changes will be independent of OPT-reactive compounds not identical with GSSG.

Mixed disulfides

Mixed disulfides were assessed by treating cell homogenates prepared as described above, with sodium borohydride. This borohydride reduction was carried out as described by De Lucia et al. [6]. Mixed disulfide levels can be calculated, as the difference in the total glutathione level of the borohydride treated preparation and an untreated control sample.

Glutathione efflux

When GSH and GSSG levels had to be measured in the covering medium, cells were exposed to ozone in the presence of a salt solution (sol A) containing 5.5 mM glucose/125 mM NaCl/5 mM KCl/2.5 mM Na_2HPO_4/2.5 mM $CaCl_2$/1.2 mM $MgSO_4$ and 17 mM HEPES (pH 7.4) instead of culture medium without serum.

This was necessary because components of the medium like phenol red, and some amino acids, present at relatively high concentrations compared to the expected small amounts of GSH and GSSG, may disturb the measurements [11], and secondly, to prevent the synthesis of extra glutathione from amino acid precursors in the medium during the time of exposure [15].

Glutathione depleted cells

A549 cells were depleted of glutathione by treating them with methionine sulfoximine (MSO) and diethylmaleate (DEM) as follows; A549 cells were grown for 3 days in petriperm dishes as described above, in the presence of 5.0 mM MSO (Sigma, St. Louis, U.S.A.), which is known to inhibit γ-glutamyl cysteine synthetase [16]. That MSO may also inhibit glutamine synthetase [16] is not of importance, because the glutamine concentration in the medium was 1.0 mM.

One hour before exposure of these cells to ozone, sol A supplemented with 5.0 mM MSO and 0.16 mM DEM (Riedel-de Haën, Hannover, F.R.G.) was added to the cells. DEM is known to react with intracellular GSH [17]. Sol A instead of medium was used for the same reasons as mentioned above.

GSH peroxidase activity

GSH peroxidase activity was measured according to Lawrence and Burk [18] in 50 mM imidazole buffer (pH 7.0) and with hydrogen peroxide as substrate.

Cell homogenates were prepared as described above.

Statistical analysis of data

Data are presented as mean ± standard error of the mean, and statistical analysis was carried out using the paired Student's *t*-test.

RESULTS

Reduction of glutathione levels of cells exposed to ozone

After exposure of cells to ozone, cellular levels of both GSH and GSSG

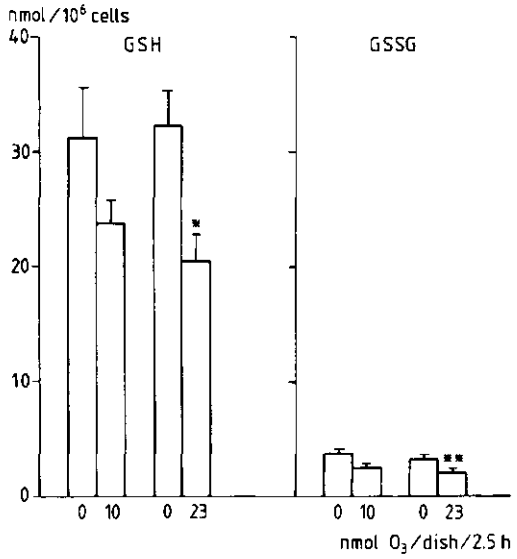

Fig. 1. Effect of ozone on the glutathione levels of A549 cells, exposed to 10 ± 2 nmol O_3/dish/2.5 h (n = 4) or to 23 ± 2 nmol O_3/dish/2.5 h (n = 5). *$P < 0.05$; **$P < 0.01$.

were reduced by approximately 20—35% in A549 cells (Fig. 1). Incubation of a homogenate of ozone exposed A549 cells with borohydride, known to release GSH from mixed disulfides, did not result in the recovery of the glutathione lost from the cytoplasm of exposed cells (Fig. 2). This is in

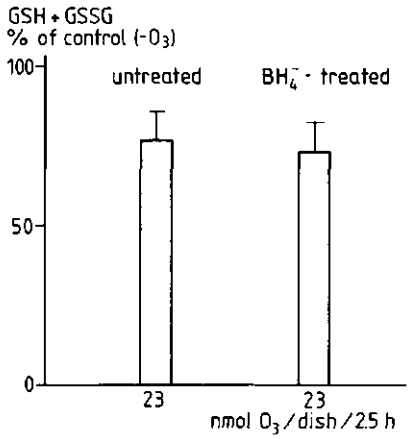

Fig. 2. Determination of glutathione incorporation into mixed disulfides during exposure of A549 cells to ozone. Cells were exposed to 23 ± 2 nmol O_3/dish/2.5 h (n = 3). Half of each homogenate was incubated with borohydride (= BH_4^--treated the other was incubated at 37°C without BH_4^- (= untreated).

TABLE I

GLUTATHIONE LEVELS IN OZONE EXPOSED AND UNEXPOSED A549 CELLS AND IN THE CULTURE MEDIUM

Ozone dose was 29 ± 2 nmol/dish/2.5 h (n = 5).

Parameter	Sample ± O_3	In the cells/dish[a]	In the medium/dish	Total/ dish
GSH	–	28.4 ± 3.1	6.1 ± 1.5	34.5 ± 2.9
In nmol	+ O_3	15.6 ± 4.4**	13.3 ± 2.7**	28.9 ± 2.9**
GSSG	–	2.8 ± 0.5	5.1 ± 0.6	7.9 ± 0.8
In nmol	+ O_3	1.3 ± 0.5***	8.4 ± 0.4***	9.7 ± 0.7**
GSH + GSSG	–	34.0 ± 3.6	16.3 ± 2.4	50.3 ± 3.6
In nmol GSH equivalents	+ O_3	18.2 ± 5.2**	30.1 ± 3.1***	48.3 ± 3.8 n.s.

[a]Calculated as 10^6 cells/dish × nmol glutathione/10^6 cells.
**$P < 0.01$.
***$P < 0.001$.

accordance with the observation presented in Table I, that almost the whole decrease in the level of GSH and GSSG in the cells could be accounted for by the observed increase in GSH and GSSG in the medium covering the cells. A considerably lower but still substantial efflux of both glutathione forms was observed for the control A549 cells (Table I). Furthermore, when the amount of trypan blue excluding cells was compared to the cellular remainder of GSH and GSSG — all parameters expressed as percentages of these values in control cells (Fig. 3) — it can be seen that even if all GSH and GSSG had leaked out of the cells that failed to exclude trypan blue, this would not account for the observed decline in cellular levels of GSH and GSSG.

Ozone sensitivity of glutathione depleted cells

In order to examine the possible role of glutathione in the defense mechanism of cells against damage by ozone, the ozone sensitivity of cells, whose glutathione level was reduced by incubation with MSO and DEM (Fig. 4a), was compared to the ozone sensitivity of untreated cells. MSO/DEM treated control cells did not differ from untreated, unexposed A549 cells with respect to their light microscopical appearance or trypan blue exclusion (data not shown), but from Fig. 4b it can be seen that glutathione depleted cells showed an increased sensitivity towards ozone. Cell survival amounted to 86% in the untreated as compared to 36% in the MSO/DEM treated cells. The trypan blue exclusion figures amounted to 91% and 56%, respectively.

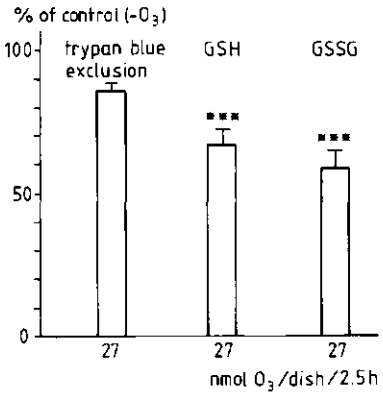

Fig. 3. Comparison of trypan blue exclusion and glutathione left in A549 cells after exposure to ozone (27 ± 2 nmol O_3/dish/2.5 h) (n = 13). ***$P < 0.001$.

Glutathione peroxidase

No activity of glutathione peroxidase, which is supposed to catalyse the detoxification of fatty acid hydroperoxides [5], could be detected in the A549 cells.

DISCUSSION

A549 cells appeared to release both GSH and GSSG to the extracellular space, a phenomenon also observed in other cultured cell types like isolated hepatocytes [19], erythrocytes [20], cultured human lymphoid cells [21] and human diploid fibroblasts [22]. This cellular release of glutathione is considered to be a normal cellular function, probably representing the initial step in the degradation of glutathione [23].

Exposure of cells to ozone resulted in a significant reduction in the cellular level of both GSH and GSSG, mainly caused by an increased efflux of both forms of glutathione out of the exposed cells, and not by the incorporation of GSH into mixed disulfides.

The mechanism of the increased efflux of GSH and GSSG from ozone exposed cells is still unclear. The following hypotheses may be envisaged: (a) leakage of both GSH and GSSG through damaged cell membranes; (b) transport of both GSH and GSSG; and/or (c) the enhanced excretion of only one of the glutathione forms, the other being lowered because the intracellular GSH/GSSG ratio was balanced.

It cannot be excluded that the reduction in cellular glutathione is completely caused by the leakage out of damaged cells. However, the amount of trypan blue excluding cells was significantly higher than the cellular remainder of GSH and GSSG after exposure of the cells to ozone. From this it

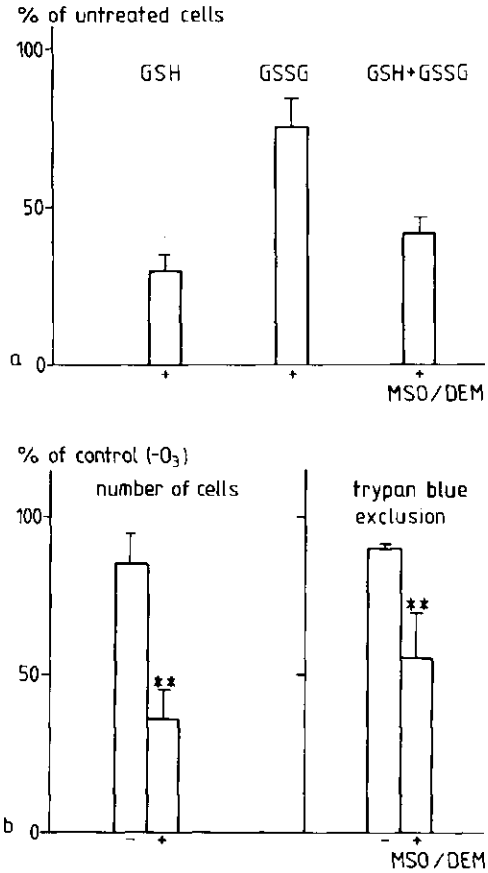

Fig. 4. (a) Percentages of glutathione left in A549 cells after treatment with MSO and DEM (n = 4). (b) Sensitivity of glutathione depleted (= MSO/DEM treated) and untreated A549 cells towards ozone. The ozone dose amounted to 25 ± 2 nmol O_3/dish/2.5 h (n = 4). Cell survival and trypan blue exclusion are presented as percentages of unexposed controls. $^{**}P < 0.02$.

was concluded that even if all GSH and GSSG had leaked out of cells that failed to exclude trypan blue, this would not account for the observed decline in cellular levels of both forms of glutathione. And this observation provides some support to the hypothesis that at least part of the reduction in cellular glutathione levels after exposure to ozone must be ascribed to a specific transport mechanism.

In several studies reported in the literature [24] an increased release of GSSG from cells exposed to "oxidative stress" factors other than ozone (e.g. H_2O_2, organic peroxides) was found. This release might be mediated by

some active transport mechanism [20]. Such an increased efflux of GSSG from cells exposed to "oxidative stress" is consistent with the results obtained in this study. The increase in GSSG in the medium covering the ozone exposed cells was higher than might be expected if both glutathione forms had leaked out of the cells proportional to their intracellular ratio.

It has also been suggested that the increased GSSG efflux from cells under oxidative stress might be a consequence of increased intracellular GSSG production by the action of glutathione peroxidase [19; 24]. With respect to ozone, such a mechanism of glutathione efflux might correspond very well with the mechanism described by Chow and Tappel [5], which implies that glutathione becomes oxidized in the glutathione peroxidase catalysed reduction of toxic fatty acid hydroperoxides, formed as a consequence of ozone induced lipid peroxidation.

However, the data reported in the present study do not support this hypothesis as A549 cells do not contain a detectable amount of glutathione peroxidase activity.

Therefore the present study does not provide support for the mechanisms proposed in the literature and referred to above: no mixed disulfides could be detected after exposure of the cells to ozone, and the observed role for intracellular glutathione in the protection of A549 cells against ozone could *not* be ascribed to the GSH peroxidase catalysed reduction of lipid peroxides. This is in accordance with several reports in literature which show that the increased activities of the enzymes of the glutathione system do not necessarily run parallel to the resistance of animals to ozone [25].

Since it was clearly demonstrated by the increased sensitivity of MSO/DEM-treated A549 cells, that glutathione plays a role in the defense mechanism of cells against the toxic action of ozone, the results are compatible with the hypotheses that : (i) glutathione acts as a direct scavenger of ozone or its oxidative initial product(s); and/or that (ii) glutathione is a prerequisite for the regeneration of other cell components, oxidized as a consequence of exposure to ozone. Such a component might be vitamin-E [9], which was shown to protect cells in vitro from intoxication by ozone in previous experiments [26].

To elucidate the observed role for glutathione in the defense mechanism of cells against ozone toxicity, studies are continued.

ACKNOWLEDGEMENTS

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CHAPTER 8

A STUDY ON THE MECHANISM OF GLUTATHIONE MEDIATED PROTECTION IN LUNG CELLS EXPOSED TO OZONE *IN VITRO*

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ABSTRACT

The results of the present study demonstrate that the protective role of glutathione in A549 cells exposed to ozone in vitro is not mediated by a glutathione dependent regeneration of oxidized α-tocopherol (vitamine E).

This conclusion is based on the following results.

First, glutathione depleted cells did not show a decreased cellular level of α-tocopherol after ozone exposure. Secondly, vitamin E supplementation did not diminish the increased ozone sensitivity of glutathione depleted cells. Furthermore the increased ozone sensitivity of glutathione depleted cells was less pronounced after cellular vitamin C supplementation.

The above data indicate that part of the protective role of glutathione is achieved by its action as a direct scavenger of reactive initial and/or intermediate species.

Additional experiments in which cellular levels of reduced (GSH) and oxidized (GSSG) glutathione were determined in control and ozone

exposed cells, either pretreated or untreated with a specific inhibitor of glutathione reductase, BCNU (= 1,3-bis(2-chloroethyl)-1-nitrosourea), demonstrated that the scavenging action of glutathione in ozone exposed cells is not accompanied by oxidation of a substantial percentage of intracellular GSH to GSSG.

Hypotheses for the mechanism of glutathione dependent scavenging of reactive intermediates, which are compatible with the results presented are **i)** the involvement of few reactive intermediates with regard to the cellular GSH content in ozone induced cell damage, resulting in undetectable GSSG formation or **ii)** the regeneration of the glutathione free radical $GS^{\cdot}$ by other cellular electron donors to GS^{-} and its protonated form GSH, which excludes its recombination with another $GS^{\cdot}$ to give GSSG.

INTRODUCTION

Glutathione (GSH) is one of the cellular reducing substances and plays a role in the defense mechanism of cells exposed to ozone. This follows from the observation that cells artificially depleted of glutathione, show an increased sensitivity towards ozone (Rietjens et al., 1985a).

Several hypotheses for the protective action of GSH against ozone have been proposed in the literature. These include **i)** glutathione peroxidase catalyzed detoxification of fatty acid hydroperoxides, possibly formed upon ozone induced lipid oxidation (Chow and Tappel, 1973), **ii)** formation of mixed disulfides between glutathione and cellular sulfhydryl groups, which may prevent the irreversible oxidation of these sulfhydryl groups by ozone (De Lucia et al., 1975), **iii)** glutathione dependent regeneration of vitamin E (α-tocopherol), possibly oxidized after the scavenging of reactive free radicals and/or other ozone intermediates (Menzel, 1970; Pryor, 1976; Mustafa and Tierney, 1978) and **iv)** a direct scavenging of reactive initial and/or inter-

mediate products by glutathione (Rietjens et al. 1985a).
Previous experiments demonstrated that the glutathione dependent protection of cells against ozone cannot be ascribed to the glutathione peroxidase catalyzed reaction, as **i)** A549 cells, which show an increased ozone sensitivity upon glutathione depletion, do not contain a detectable glutathione peroxidase activity (Rietjens et al., 1985a) and **ii)** alveolar macrophages and type II cells, isolated from the lungs of ozone exposed rats did not show an increased resistance towards ozone in spite of a markedly increased glutathione peroxidase activity (Rietjens et al., 1985b).
In addition ozone exposure was not accompanied by a significant incorporation of glutathione into mixed disulfides (Rietjens et al., 1985a). Therefore the protective action of glutathione may be best explained by hypotheses **iii)** and **iv)** mentioned above.
The objective of the present study was to determine the contribution of each of these hypothetical mechanisms to the glutathione mediated protection of ozone exposed cells.

MATERIALS AND METHODS

Cell culture

Cells of the alveolar type II cell-line A549 were cultured in Ham's F10 medium (Flow, Irvine, Scotland), supplemented with $NaHCO_3$ (1.2 g/l), 10% newborn calf serum (Gibco, Glasgow, Scotland), 50 IU/l of penicillin (Gist-Brocades, Delft, The Netherlands), and 50 mg/l streptomycin (Specia, Paris, France) in 75 cm^2 tissue culture flasks (Costar, Cambridge, Mass. USA) at 37°C in a humid atmosphere containing 5% CO_2 in air. Before use, cells were dislodged with a solution containing 0.25% trypsin (Difco, Detroit, Mich. USA) and 0.05% EDTA (Merck, Darmstadt, West Germany).
For each experiment approximately 2.10^5 cells were plated onto a plastic film dish (Petriperm, Heraeus, Hanau, West Germany) with a 25

μm teflon bottom and a growth area of 18 cm^2. Cells were cultured for 3 days until a confluent monolayer was obtained.

Exposure to ozone

Cells were exposed to ozone as described before (Alink et al., 1979; Rietjens et al., 1985a) in the presence of a balanced salt solution (sol A) containing 5.5 mM glucose/125 mM NaCl/5 mM KCl/2.5 mM Na_2HPO_4/2.5 mM $CaCl_2$/1.2 mM $MgSO_4$ and 17 mM HEPES pH 7.4.
The amount of ozone which diffused through the teflon film into the dish was measured using the indigo disulfonic acid method (Guicherit et al., 1972).

Parameter for ozone toxicity

Cell survival, expressed as percentage of the unexposed control, was calculated as the product of viability and cell number. Viability of the cells was assessed by counting the number of trypan blue excluding cells out of 400, 5-10 minutes after addition of trypan blue (BDH Chemicals Poole, England) (0.5% final concentration).

Biochemical Parameters

GSH and GSSG were measured as described before (Rietjens et al., 1985a), by the method of Hissin and Hilf (1976).
Glutathione reductase (GR) activity was measured as described by Racker (1955). Cells were disrupted by freezing in liquid nitrogen and thawing at 37°C, which was repeated twice.
Glucose 6-P-dehydrogenase (G6PDH) activity was measured using a Boehringer test-combination (Boehringer, Mannheim, West Germany).

Modification of cellular levels of glutathione, glutathione reductase, vitamin E or vitamin C.

A549 cells were depleted of glutathione by treating them with methionine sulfoximine (MSO) (Sigma, St. Louis, USA) and diethyl-maleate (DEM) (Riedel-de Haën, Hannover, West Germany). A549 cells

were grown for 3 days in petriperm dishes in the presence of 5.0 mM MSO, which is known to inhibit γ-glutamyl-cysteine synthetase (Meister, 1983). - That MSO may also inhibit glutamine synthetase is not of importance, because the glutamine concentration in the medium was 1.0 mM -. Before exposure to ozone, cells were preincubated for 2.5 h with 5.0 mM MSO and 0.15 mM DEM in sol A, washed and exposed to ozone in the presence of sol A without additional MSO and DEM. DEM is known to react with intracellular GSH (Boyland and Chasseaud, 1967). Incubation of A549 cells with MSO/DEM was shown to reduce cellular glutathione levels significantly (Rietjens et al., 1985a).

Cellular GR activity was inhibited by treating the cells with 0.1 mM 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)(Bristol-Meyers, Syracuse, New York, USA) in sol A, for 30 minutes at 37°C. BCNU is known to cause a relatively selective, irreversible and almost complete deficiency of cellular GR activity (Frisher and Ahmad, 1977; Shinohara and Tanaka, 1979; McKenna et al. 1983; Eklöw et al., 1984). Inhibition seems to be caused by binding of an isocyanate derivative to the thiol group(s) at the active site of the enzyme (Babson and Reed, 1978). Cells were washed and exposed to ozone in the presence of sol A without additional BCNU.

Cellular levels of α-tocopherol (vitamin E) were increased by culturing the cells in the presence of vitamin E for 2 days. A stock solution of Dl- α-tocopherol (Merck, Darmstadt, West Germany) was made in ethanol (10 mg/ml) and diluted in Ham's F10 medium. Before exposure, cells were preincubated for 2.5 h in the presence of vitamin E in sol A, washed and then exposed to ozone in the presence of sol A without additional vitamin E.

Cellular levels of ascorbic acid (vitamin C) were increased by incubating the cells for 2.5 h at 37°C in the presence of L-ascorbic acid (Merck, Darmstadt, West Germany) in sol A at pH 7.4. Cells were washed

and exposed to ozone in the presence of sol A without additional vitamin C.

α -Tocopherol content of control and glutathione depleted cells

The effect of ozone on cellular α-tocopherol levels was determined in A549 cells preincubated for 2 days with 23 μM α-tocopherol and treated or not with MSO/DEM to deplete cellular glutathione levels. The cells were exposed to ozone in the presence of 10 ml sol A and collected after exposure by scraping them from the dishes. Cells were disrupted by freezing in liquid nitrogen and thawing at 37°C which was repeated twice. α-Tocopherol was extracted from the pooled cell samples of two dishes (2 ml) with 9.5 ml chloroform: MeOH (2:1), following the method described by Folch et al. (1957) and Desai (1980). Reduced α-tocopherol was measured fluorometrically in ethanol with λexcitation = 295 nm and λemission = 340 nm (Duggan, 1959; Desai, 1980).

The validity of this method for the measurement of diminished levels of cellular α-tocopherol has been demonstrated recently by the detection of significantly reduced α-tocopherol levels in nitrogen dioxide exposed cells. (Rietjens et al. in press).

Statistical analysis of data

Data are presented as mean ± standard error of the mean, and statistical analysis was carried out using the paired Student's t-test.

RESULTS

Effect of ozone on α -tocopherol in normal and glutathione depleted A549 cells

From the results presented in Table 1 it follows that exposure of A549 cells to ozone did not affect their cellular α-tocopherol content.

Moreover, glutathione depleted cells also did not lose a detectable amount of their α-tocopherol content during exposure to ozone (Table 1).

TABLE 1. EFFECT OF OZONE (35 ± 2 NMOL O_3/DISH) ON THE α-TOCOPHEROL CONTENT OF CONTROL AND GLUTATHIONE DEPLETED (= MSO/DEM-TREATED) A549 CELLS. All cells were preincubated with 23 μM α-tocopherol for 2 days to achieve a detectable cellular α-tocopherol content. n.s. = not significant

cells	nmol α-tocopherol / 10^6 cells		
	$-O_3$	$+O_3$	
control	1.62 ± 0.13	1.50 ± 0.14	n.s.
glutathione depleted	1.89 ± 0.22	1.88 ± 0.16	n.s.

Influence of vitamin E or vitamin C supplementation on the increased ozone sensitivity of glutathione depleted cells

From Figure 1 it can be seen that vitamin E protected both glutathione depleted and control cells from damage caused by ozone. By increasing the vitamin E concentration of the preincubation medium from 0.5 to 116 μM, cell survival after ozone exposure was increased from 55 ± 8% to 90 ± 4% ($p < 0.01$) for control and from 21 ± 5% to 59 ± 6% ($p < 0.01$) for glutathione depleted cells.

However, the difference in ozone sensitivity between untreated and glutathione depleted cells could not be reduced by elevation of the cellular vitamin E content. The difference in cell survival between control and glutathione depleted cells amounted to 34%, 34%, 29% and 31% at 0.5, 0.8, 23.2 and 116 μM vitamin E respectively.

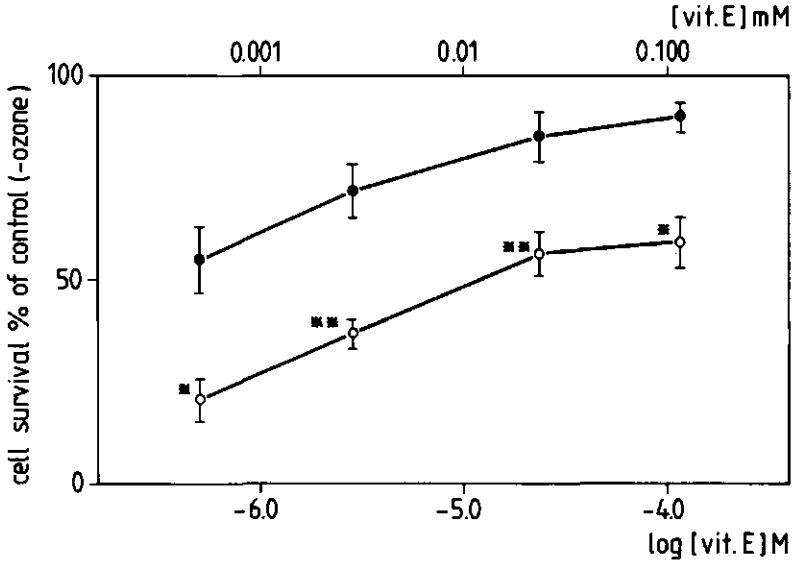

Figure 1. Protective action of vitamin E on control (-●-) and glutathione depleted (-o-) A549 cells, exposed to ozone (16 ± 1 nmol O_3/dish).
** = $p < 0.01$, * = $p < 0.05$ (n = 6).

Vitamin C also protected both normal and glutathione depleted cells from damage caused by ozone (Figure 2). By raising the vitamin C concentration during the preincubation from 0.001 to 10 mM, cell survival after exposure to ozone was increased from 58 ±4% to 88 ±3% ($p < 0.01$) for control and from 28 ± 7% to 77 ± 4% ($p < 0.01$) for glutathione depleted cells.

Thus, in contrast to vitamin E, vitamin C enrichment of the cells partly abolished the increased ozone sensitivity of glutathione depleted cells (Figure 2). At vitamin C concentrations of 0.001, 0.01, 1 and 10 mM, the difference in cell survival after ozone exposure between control and glutathione depleted cells amounted to 30%, 30%, 15% and 11%, respectively. The difference of 11% at 10 mM vitamin C was significantly smaller than the 30% at 0.001 mM ($p < 0.05$).

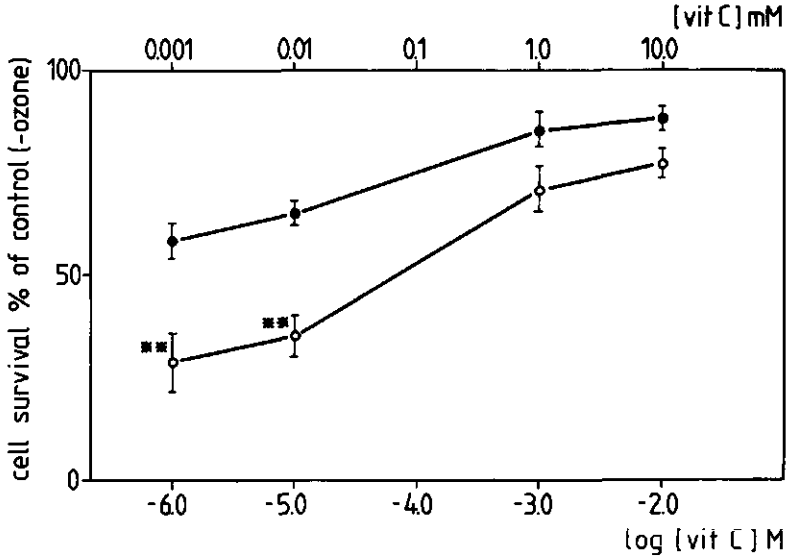

Figure 2. Protective action of vitamin C on control (-●-) and glutathione depleted (-o-) A549 cells exposed to ozone (18 ± 2 nmol O_3/dish). ** = $p < 0.01$ ($n = 6$).

The data presented in Table 2 demonstrate that vitamin C supplementation of the cells does not affect the level of glutathione depletion in these cells by MSO/DEM treatment. The percentage of GSH left after MSO/DEM treatment amounted to 42% for control (0.001 mM vit C in preincubation) and 41% for vitamin C supplemented cells (10 mM vit C in preincubation). For GSSG these values amounted to 81% and 72% respectively.

Influence of ozone on A549 cells with inactivated glutathione reductase (GR)

A549 cells treated with BCNU showed a marked decrease in their GR activity: GR activity in BCNU treated cells amounted to only 5% ($p < 0.001$) of the activity in untreated control cells (Table 3). The

TABLE 2. INFLUENCE OF VITAMIN C PREINCUBATION (2.5 hour, 0.001 mM = control or 10 mM = vit C supplemented), ON GLUTATHIONE DEPLETION IN A549 CELLS, ACHIEVED BY INCUBATION WITH MSO/DEM.
** $p < 0.01$, * $p < 0.05$, n.s. = not significant ($n = 6$)

parameter	control (0.001 mM vit C)	vit C supplemented (10 mM vit C)	
nmol GSH/10^6 cells			
control	25.0 ± 2.6	21.3 ± 2.0	n.s.
glutathione depleted	10.3 ± 2.6	8.7 ± 0.8	n.s.
(= MSO/DEM-treated)	**	**	
nmol GSSG/10^6 cells			
control	3.7 ± 0.5	4.7 ± 0.5	n.s.
glutathione depleted	3.0 ± 0.5	3.4 ± 0.4	n.s.
(= MSO/DEM-treated)	n.s.	*	

cellular level of reduced glutathione and the activity of G6PDH, an enzyme which also has a thiol group at the active site, showed no significant change after incubation of the cells with BCNU (Table 3).

From the results presented in Table 3 it can be seen that the BCNU treated cells showed no increased sensitivity towards ozone. Furthermore, the changes in cellular GSH and GSSG levels after exposure of the cells to ozone, were the same for both BCNU treated and untreated control cells (Table 3). Neither in control, nor in BCNU treated cells a substantial intracellular oxidation of GSH to GSSG after exposure to ozone was found (Table 3).

TABLE 3. SPECIFIC INHIBITION OF GLUTATHIONE REDUCTASE (GR) ACTIVITY OF A549 CELLS BY TREATMENT WITH 0.1 mM 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA (BCNU) FOR 30 MINUTES AT 37°C, AND THE EFFECT OF SUBSEQUENT OZONE EXPOSURE (16 ± 2 NMOL O_3/DISH) ON GLUTATHIONE LEVELS AND SURVIVAL OF THESE CELLS WITH INACTIVATED GR.
*** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$, n.s. = not significant (n = 6)

parameter	± O_3	control cells	BCNU treated cells	
GR				
µmol NADPH oxidized/ min/10^8 cells	-	1.89 ± 0.06	0.09 ± 0.01	***
G6PDH				
µmol NADPH reduced/ min/10^7 cells	-	1.92 ± 0.12	1.90 ± 0.08	n.s.
GSH	-	22.8 ± 3.3	21.7 ± 3.9	n.s.
nmol/10^6 cells	+ O_3	10.2 ± 1.4 **	10.3 ± 2.0 **	n.s.
GSSG	-	1.8 ± 0.2	1.6 ± 0.2	n.s.
nmol/10^6 cells	+ O_3	1.1 ± 0.1 *	1.0 ± 0.1 *	n.s.
cell survival after O_3 exposure % of control (= $-O_3$)	+ O_3	35.8 ± 2.6	37.2 ± 5.9	n.s.

DISCUSSION

Glutathione depleted A549 cells demonstrated an increased sensitivity towards ozone, a phenomenon reported before, and demonstrating the involvement of glutathione in the cellular defense against ozone (Rietjens et al., 1985a).

Previous results (Rietjens et al., 1985a) also demonstrated that the

protective role of glutathione could not be ascribed to the glutathione peroxidase catalyzed detoxification of lipid hydroperoxides as suggested by Chow and Tappel (1973), or to formation of mixed disulfides protecting thiol groups against irreversible oxidation by ozone, as was put forward by De Lucia et al. (1975).

As shown in the present study the increased ozone sensitivity of glutathione depleted cells can also not be ascribed to an impairment of a possible glutathione mediated α-tocopherol regeneration in these cells. This eliminates a third hypothesis for glutathione dependent protection against ozone. Clear evidence for this conclusion follows from the observation that the α-tocopherol level of glutathione depleted cells was not affected following exposure of the cells towards ozone. A second line of evidence followed from the observation that vitamin E supplementation of glutathione depleted cells did not diminish the increased ozone sensitivity of the cells. Both phenomena should have been observed when an impaired regeneration of α-tocopherol, resulting in a reduced cellular level of the antioxidant, would have been the main cause of the increased ozone sensitivity of glutathione depleted cells.

Recently Hill and Burk (1984), using an ascorbate iron microsomal lipid peroxidation system, presented evidence from which it was concluded that a GSH dependent radical scavenging system and α-tocopherol function independently to protect the microsomal membrane from free radical attack. Their findings are compatible with the results of the present study.

In contrast to vitamin E supplementation cellular vitamin C supplementation resulted in a decreased difference in cell survival after ozone exposure between control and glutathione depleted cells. This indicates that part of the antioxidant function of glutathione in lung cells exposed to ozone could be taken over by another cellular water soluble antioxidant, vitamin C.

The foregoing results strongly suggest that the protective action of

glutathione in ozone exposed cells is achieved by its action as a direct scavenger of reactive initial and/or intermediate species (hypothesis iv).

This scavenging action of glutathione may be accompanied by its oxidation to the disulfide form GSSG. This could occur either i) by combination of the glutathione free radical GS$^{\cdot}$ - formed upon the reaction of GSH with a free radical intermediate -, with another GS$^{\cdot}$ (Pryor, 1976) and/or by ii) direct oxidation of GSH by ozone (Menzel, 1971) or perhaps an ozonide intermediate (Menzel et al., 1975).

In this study GSH and GSSG levels were determined in control and ozone exposed cells either treated or untreated with BCNU. BCNU treatment resulted in a specific 95% inactivation of the glutathione reductase activity of the cells, a phenomenon also observed in human platelets (McKenna et al., 1983) and red blood cells (Shinohara and Tanaka, 1979; Frisher and Ahmad, 1977), and in rat hepatocytes (Eklöw et al., 1984) treated with BCNU.

Such an extreme inhibition of glutathione reductase activity has been shown to result in an insufficient reduction of GSSG to GSH in the cytoplasm of cells exposed to GSSG forming, oxidative stress factors. Exposure to diamide, t-butyl hydroperoxide, benzylamine or hexobarbital resulted in a decreased intracellular GSH content and an increased intracellular and/or medium GSSG content for exposed, BCNU treated cells as compared to exposed, non-BCNU treated cells (Eklöw et al., 1984). Such a result was not observed in the present study.

Exposure to ozone led to changes in glutathione contents of BCNU treated cells that were similar to the changes in ozone exposed untreated cells: a decrease of both oxidized and reduced glutathione was observed, which is known to be caused by their leakage and/or active transport to the surrounding medium (Rietjens et al., 1985a).

Hypotheses compatible with these results, which show no increased GSSG formation in ozone exposed cells, are i) the involvement of a relatively low level of intermediates compared to the cellular GSH content in ozone induced cell damage, and thus in an undetectable GSSG

formation or ii) the regeneration of the glutathione free radical GS$^{\cdot}$ by other cellular electron donors to GS^{-} and its protonated form GSH, which excludes the formation of GSSG from GS$^{\cdot}$ with another GS$^{\cdot}$ (Forni and Willson, 1983).

In any case the present results show that ozone apparently does not impose the same type of oxidative stress on the cells as the compounds mentioned above.

ACKNOWLEDGEMENTS

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CHAPTER 9

THE ROLE OF GLUTATHIONE AND GLUTATHIONE S-TRANSFERASE IN FATTY ACID OZONIDE DETOXIFICATION

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SUMMARY

The ozonide derived from methyl linoleate was shown to cause a dose dependent inhibition of the phagocytosis of rat alveolar macrophages exposed in vitro to concentrations varying from 10^{-5} to 10^{-4} M.
Vitamin C was demonstrated to detoxify the ozonide. In analogy to their behaviour on exposure to ozone, vitamin E supplemented cells demonstrated a decreased and glutathione depleted cells an increased sensitivity towards the compound. The characteristics of antioxidant protection of cells against the ozonide were thus comparable to those for protection against ozone.
Preincubation with glutathione also detoxified the ozonide model compound. Survival of rat alveolar macrophages exposed to a toxic concentration of the ozonide (86 μM final concentration), measured by phagocytosis of the cells, increased significantly ($p < 0.01$) from 23 to 54% after a 2.5 hour preincubation of the ozonide with glutathione (5 mM final concentration).
The detoxification of methyl linoleate ozonide by glutathione could be catalyzed by the rat liver glutathione S-transferases. After a 2.5

hour preincubation of the ozonide (86 µM final concentration) with glutathione and glutathione S-transferase (final concentrations respectively 5 mM and 0.01 mg/ml), its toxicity was completely abolished, as demonstrated by the 98% survival ($p < 0.001$) of subsequently exposed cells.

A Km_{app} (at 1 mM glutathione) for the ozonide of 0.80 mM and a $Vmax_{app}$ (at pH 6.5) of 94 nmol glutathione converted $.min^{-1}.mg\ protein^{-1}$ or (at pH 7.4) of 34 nmol glutathione converted $.min^{-1}.mg\ protein^{-1}$, were found.

This glutathione S-transferase catalyzed detoxification of the potential intermediates in ozone induced cell damage, offers a completely new viewpoint on the role of glutathione in the protection of cells against ozone.

INTRODUCTION

Ozone is one of the most toxic components present in photochemical smog (1). The mechanism of its toxic action has as yet not been elucidated. It has been suggested that cell damage arises from the oxidation of the unsaturated fatty acid moieties in the membrane phospholipids (1;2;3). Oxidation of these unsaturated fatty acids may very well proceed by formation of fatty acid ozonides (1), since exposure of pure fatty acid monolayers or suspensions to ozone results in the formation of such ozonides as the major products (4;5;6).

Support for this hypothesis has been reported by Menzel et al. (7) who showed that fatty acid ozonides produce Heinz body-like inclusions in human erythrocytes following in vitro exposure, a phenomenon also found for ozone exposed whole blood and for erythrocytes from ozone exposed mice (7).

In addition Cortesi and Privett (8) reported that methyl linoleate ozonide injected intravenously, caused effects on rat lung similar in many respects to those reported for ozone. From these results it

follows that fatty acid ozonides are likely candidates for an intermediate role in the mechanism of ozone toxicity.

Several hypotheses for the protective action of glutathione in ozone exposed cells have been proposed in the literature. These include **i)** glutathione peroxidase catalyzed detoxification of fatty acid hydroperoxides, possibly formed upon ozone induced lipid oxidation (9), **ii)** formation of mixed disulfides between glutathione and cellular sulfhydryl groups, thus may be preventing their irreversible oxidation by ozone (10), **iii)** glutathione dependent regeneration of vitamin E, possibly oxidized after the scavenging of reactive free radicals and/or other ozone intermediates (2), or **iv)** a direct scavenging of reactive initial and/or intermediate products by glutathione (11).

Previous experiments demonstrated that the glutathione dependent protection of cells against ozone, could not be ascribed to the glutathione peroxidase catalyzed reaction, as **i)** A549 cells, which show an increased ozone sensitivity upon glutathione depletion, do not contain a detectable amount of glutathione peroxidase activity (11) and **ii)** alveolar macrophages and type II cells isolated from the lungs of ozone exposed rats did not show an increased resistance towards ozone in spite of a markedly increased glutathione peroxidase activity (12). In addition, ozone exposure was not accompanied by a significant incorporation of glutathione into mixed disulfides (11). Furthermore glutathione depleted cells did not contain a decreased level of vitamin E (α-tocopherol) upon ozone exposure and their increased ozone sensitivity could not be abolished by vitamin E supplementation (13). This eliminated the possible impairment of vitamin E regeneration as a mechanism for the increased ozone sensitivity of glutathione depleted cells.

Therefore, the protective action of glutathione may be best explained by its action as a direct scavenger of initial and/or intermediate reactive species (hypothesis **iv**).

The objective of the present study was to test the potential of glutathione to react with intermediates in the toxic action of ozone, using

the ozonide derived from methyl linoleate as a model compound.
In addition some characteristics of the in vitro antioxidant protection against the toxicity of the ozonide model compound were determined, and compared to known characteristics of in vitro cellular antioxidant protection against ozone, to obtain additional support for the involvement of ozonides in ozone induced cell damage.

MATERIALS AND METHODS

Synthesis of methyl linoleate ozonide

Methyl linoleate ozonide was synthesized by the procedure of D.B. Menzel (personal communication), which was described by Calabrese et al. (14;15).
In short, one gram of methyl linoleate (Sigma, St. Louis, U.S.A.) was dissolved in 20 ml pentane, flushed with nitrogen and placed on an ice bath. Liquid ozone (blue) was prepared by leading an oxygen/ozone stream, generated using an ozone generator (Fisher, model 501, Meckenheim, West Germany), through a 50 cm glass tube (ϕ = 1 cm) filled with silicagel 60 (18-35 mesh) (Mackery Nagel & Co., Duren, West Germany) and suspended in a dry-ice-isopropanol bath (-80°C).
When all silicagel had a blue appearance caused by adsorbed liquid ozone, the gas flow was switched to pure nitrogen (< 2 bubbles/sec.) for 10 minutes to sweep out the residual oxygen. Following this, the nitrogen/ozone gas stream was led through the methyl linoleate solution which was kept at 0°C. The oxidation reaction was followed using TLC (silicagel plates eluted with 5% ether in petroleum ether) and ozonization was continued until all methyl linoleate had disappeared. TLC plates were stained using potassium permanganate. The major methyl linoleate ozonide formed (Rf = 0.30) was purified from the mixture, using a silicagel 60 (70-230 mesh) (Merck, Darmstadt, West Germany) column eluted with 5% ether in petroleum ether. IR, NMR and MS data showed it to be the 9,10-mono-ozonide of methyl linoleate.

Cell culture

Alveolar macrophages were isolated from the lungs of Wistar rats (± 200 g) as described by Mason et al. (16), by the lavage of isolated perfused lungs. Approximately 10^6 cells were plated onto a culture dish with a growth area of 21 cm^2 (Costar, Cambridge, MA, U.S.A.). Alveolar macrophages were cultured in Ham's F10 medium (Flow, Irvine, Scotland) supplemented with $NaHCO_3$ (1.2 g/l), 10% newborn calf serum (Gibco, Glasgow, Scotland), 50 IU of penicillin (Gist-Brocades, Delft, The Netherlands), and 50 mg/l streptomycin (Specia, Paris, France) at 37°C in a humid atmosphere containing 5% CO_2 in air. Cells were cultured for 3 days in order to attach to and stretch on the culture dish before they were exposed to the fatty acid ozonide. During these days their cellular glutathione, vitamin E or vitamin C levels could be modified - if desired - as described below.

Modification of cellular antioxidant levels

Cellular levels of glutathione, vitamin C and vitamin E were modified using optimum preincubation conditions, determined previously (17). Briefly, alveolar macrophages were depleted of glutathione by treating them with 4.0 mM methionine sulfoximine (MSO) (Sigma, St. Louis, MO, U.S.A.) and 60 μM diethyl maleate (DEM) (Riedel-de Haen, Hannover, West Germany). Cellular levels of α-tocopherol (vitamin E) were increased by culturing the cells for 2 days in the presence of 46 μM DL- α-tocopherol (Merck, Darmstadt, West Germany). Cellular levels of ascorbic acid (vitamin C) were increased by incubating the cells for 2.5 h in the presence of 1 mM L-ascorbic acid (Merck, Darmstadt, West Germany).

Determination of phagocytosis

Phagocytosis was determined after incubation of the cells for 1.5 h at 37°C in the presence of approximately 10^7 dead yeast cells, coloured by boiling them for 30 minutes in a congo red (Fluka, Buchs, Switzerland) solution.

Exposure of cells to methyl linoleate ozonide

Appropriate dilutions (final concentrations ranging from 2.5 to 86 μM) were made, of a 1 μl/ml (2.5 mM) suspension of methyl linoleate ozonide in Ham's F10 medium without serum, prepared by sonication for 30 seconds using a Branson Sonic Power Sonicator at maximum energy output. A fresh suspension was made for each experiment in Ham's F10 medium without serum. In this suspension the ozonide was stable for at least 6 hours.

Cells were exposed for 2.5 hour at 37°C to methyl linoleate ozonide in 5 ml Ham's F10 medium without serum, except for the glutathione depleted and their control cells, which were exposed in 5 ml of a buffered salt solution containing 5.5 mM glucose/125 mM NaCl/5 mM KCl/2.5 mM Na_2HPO_4/2.5 mM $CaCl_2$/1.2 mM $MgSO_4$ and 17 mM HEPES (pH 7.4). This was done to prevent the synthesis of new glutathione from amino acid precursors in the medium during the time of exposure (18). Detoxification of methyl linoleate ozonide by vitamin C was studied by determining the toxicity of the ozonide to alveolar macrophages as described above, after a 2.5 hour preincubation of the ozonide at 37°C in the presence or absence of 1 mM vitamin C (final concentration).

Spontaneous and enzyme catalyzed detoxification of methyl linoleate ozonide by glutathione

A toxic concentration of the ozonide (86 μM final concentration) was preincubated for 2.5 hour at 37°C in the presence of 5 mM reduced glutathione (Sigma, St. Louis, MO, U.S.A.) in the presence or absence of 0.01 mg/ml glutathione S-transferase (a gift from H.J.J.M. Dahlmans; the total mixture of isoenzymes was isolated from rat liver cytosol by affinity chromatography (19)) and then added to the cells. To exclude a possible aspecific protein effect, incubations of the ozonide with 0.10 mg/ml BSA (bovine serum albumin) (Sigma, St. Louis, MO, U.S.A.) were also tested.

Determination of glutathione S-transferase activity

The glutathione S-transferase activity towards methyl linoleate ozonide was determined essentially as described by Baars et al. (20). The standard assay was carried out in 0.1 M potassium phosphate/1 mM EDTA buffer at pH 6.5 or 7.4, containing 1 mM GSH, 1.5 mg/ml glutathione S-transferase and 65 to 1300 µM of the ozonide.

The total incubation volume was 2.0 ml and the reaction was started by addition of the GSH. Incubation took place at 37°C in a shaking water bath for up to 5 minutes. At 30 second time intervals 100 µl of the incubation mix was added to 10 µl 33% aqueous trichloroacetic acid to stop the reaction, placed on ice and centrifuged for 3 minutes. The amount of unreacted GSH in the clear supernatant was determined with Ellman's reagent (0.5 mM 5,5'-dithio-bis-2-nitrobenzoic acid in 0.1 M potassium phosphate buffer at pH 6.5); 50 µl of the supernatant was added to 0.95 ml of the reagent and after 5 minutes at room temperature the absorption at 412 nm was measured. Using an appropriate calibration curve the amount of unreacted glutathione in the incubation mix could be calculated. The glutathione S-transferase activity in the assay followed from the time dependent decrease in the amount of unreacted glutathione. Under the above mentioned assay conditions non-enzymatic reactions between glutathione and methyl linoleate ozonide were negligible. Apparent Km and Vmax values were calculated from Lineweaver-Burk plots.

Statistical analysis of data

Data are presented as mean $\pm$ standard error of the mean, and statistical analysis was carried out using the paired Student's t-test.

RESULTS

Methyl linoleate ozonide, toxicity and characteristics of cellular antioxidant protection

The data presented in Figure 1 show that both methyl linoleate itself and the ozonide of methyl linoleate caused a dose dependent decrease in the phagocytosis of alveolar macrophages. Methyl linoleate ozonide was about 1000 times more toxic than the original fatty acid methyl ester.

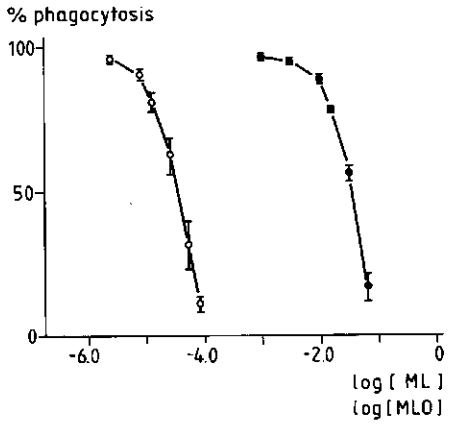


Figure 1. Toxicity of methyl linoleate (ML) (-●-) and methyl linoleate ozonide (MLO) (-o-) towards rat alveolar macrophages. Experiments were performed as described in Materials and Methods. Concentrations are in M.

Figure 2 shows the effect of modification of several cellular antioxidant systems on the cellular sensitivity towards the ozonide of methyl linoleate. Glutathione depleted alveolar macrophages demonstrated an increased sensitivity towards the ozonide (Figure 2a) whereas supplementation of the cellular α-tocopherol (vitamin E) content protected the cells from damage (Figure 2b).

Preincubation of the cells with vitamin C resulted in very little protection against methyl linoleate ozonide (Figure 2c). On the other

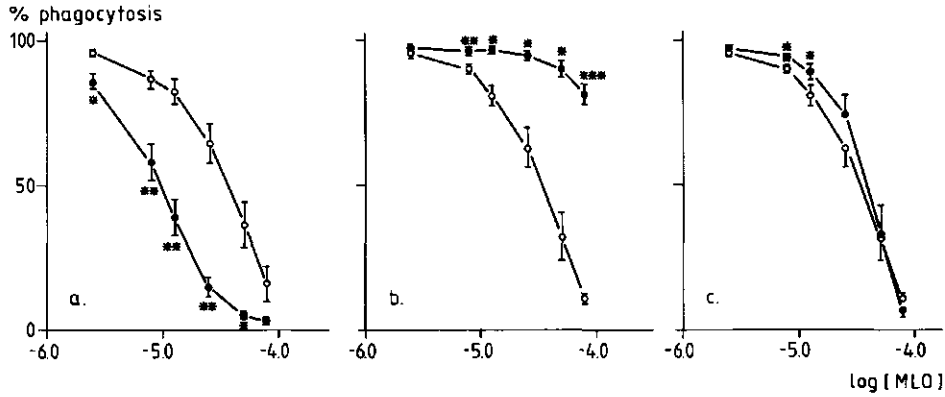


Figure 2. Effect of cellular a) glutathione depletion, b) vitamin E supplementation and c) vitamin C supplementation, on the sensitivity of alveolar macrophages to methyl linoleate ozonide (MLO). Modification of cellular antioxidant levels was carried out as described in Materials and Methods. The concentration of MLO is in M.
* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ (n = 5).

hand, from the data depicted in Figure 3 it can be seen that preincubation of the ozonide itself with vitamin C resulted in a significant detoxification of the compound.

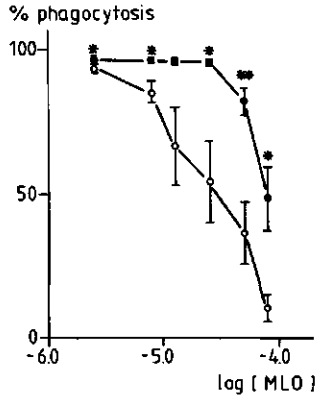


Figure 3. Detoxification of methyl linoleate ozonide (MLO) (in M) by preincubation of the ozonide with vitamin C. Following a 2.5 hour preincubation at 37°C in the presence (-●-) or absence (-o-) of 1 mM vitamin C (final concentration), toxicity towards alveolar macrophages was determined.
* = $p < 0.05$, ** = $p < 0.01$ (n = 5).

Detoxification of methyl linoleate ozonide by glutathione and glutathione S-transferase

From the results presented in Figure 4 it can be seen that preincubation of methyl linoleate ozonide with reduced glutathione caused a significant detoxification of the fatty acid ozonide. In addition, rat liver glutathione S-transferase was shown to catalyze the glutathione mediated detoxification of the ozonide, since preincubation of the ozonide with both glutathione and glutathione S-transferase completely abolished its toxicity (Figure 4). Control incubations of the ozonide with glutathione S-transferase alone, and with bovine serum albumin - with and without glutathione -, demonstrated that the complete detoxification of methyl linoleate ozonide by glutathione S-transferase plus glutathione as compared to glutathione alone, is not caused by an aspecific protein effect (Figure 4).

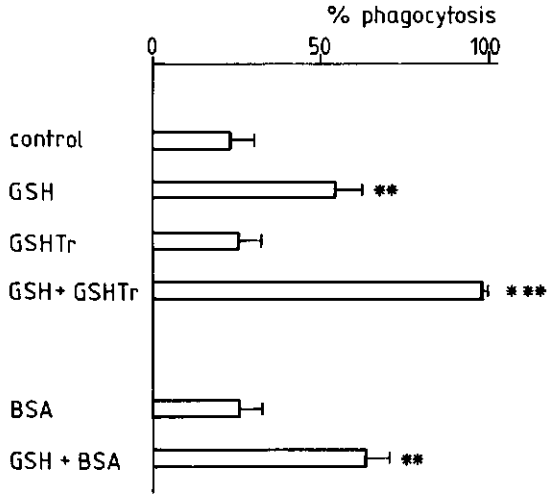


Figure 4. Detoxification of methyl linoleate ozonide (86 μM) by preincubation of the ozonide with glutathione (GSH) (1 mM), glutathione S-transferase (GSHTr) (0.01 mg/ml) or both. Control incubations with bovine serum albumin (BSA) were carried out using 0.10 mg BSA/ml. Following a 2.5 hour preincubation at 37°C, toxicity towards alveolar macrophages was determined.

** = $p < 0.01$, *** = $p < 0.001$ ($n = 5$).

Furthermore, additional experiments demonstrated that the ozonide was stable and did not lose any of its toxicity during the 2.5 hour at 37°C, and also that preincubation of the alveolar macrophages with glutathione did not result in an increased cellular resistance towards the ozonide. This indicates that the disappearance of the toxicity of methyl linoleate ozonide was indeed caused by a detoxification reaction occurring in the preincubation mix.

Enzymatic characteristics of the glutathione S-transferase catalyzed reaction between glutathione and methyl linoleate ozonide

The enzymatic characteristics of the glutathione S-transferase catalyzed reaction between methyl linoleate ozonide and glutathione were next investigated. The results are presented in Figure 5. The rate of the reaction was linear with time up to 5 minutes. From the Lineweaver-Burk plots obtained by varying the ozonide concentration at a glutathione concentration of 1 mM, a Km_{app} for the ozonide of 0.80 mM and a $Vmax_{app}$ of 94 nmol glutathione converted.min^{-1}.mg $protein^{-1}$ were determined at pH 6.5 (Figure 5a). At pH 7.4 (Figure 5b) these values amounted to Km_{app} = 0.80 mM and $Vmax_{app}$ = 34 nmol glutathione converted.min^{-1}.mg $protein^{-1}$.

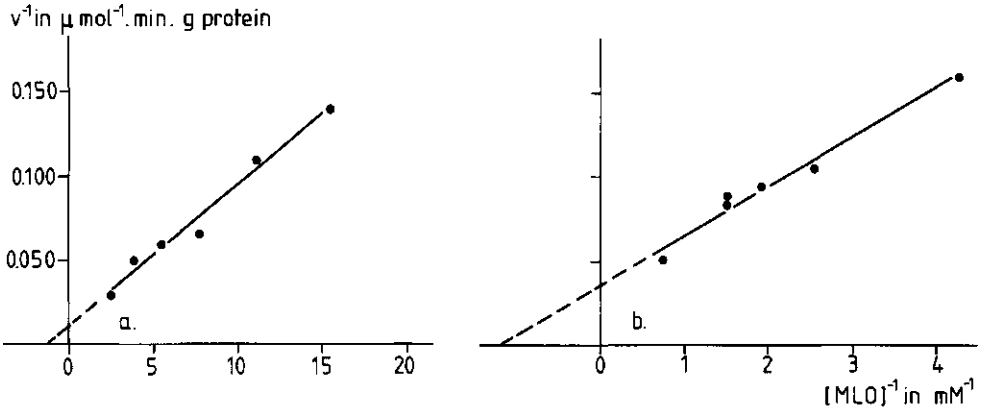


Figure 5. Lineweaver-Burk plots of the glutathione S-transferase catalyzed reaction between methyl linoleate ozonide (MLO) at different concentrations, and glutathione at 1 mM, determined at a) pH = 6.5 and b) pH = 7.4.

DISCUSSION

The toxicity of the ozonide of methyl linoleate, a possible intermediate in the toxic effects of ozone (7;8;21), was demonstrated using rat alveolar macrophages. The ozonide was 1000 times more toxic than the fatty acid methyl ester itself, and caused a dose dependent inhibition of the phagocytosis of rat alveolar macrophages exposed in vitro to concentrations varying from 10^{-5} to 10^{-4} M. Glutathione depleted alveolar macrophages showed an increased and vitamin E supplemented cells a decreased sensitivity towards methyl linoleate ozonide, phenomena comparable to changes in the ozone sensitivity of these cells (17).

Preincubation of alveolar macrophages with vitamin C, however, did not result in a significant protection against the ozonide, although vitamin C supplemented cells showed a reduced sensitivity to ozone itself (17). This discrepancy can at present not be explained except by supposing that the ozonides formed in the cell membrane during exposure to ozone have a better chance of being detoxified by cellular vitamin C than the ozonides that are added to the cells from the outside. In any case, on preincubation of the ozonide with vitamin C a significant detoxification was observed.

Taken together the results of antioxidant protection of the cells against methyl linoleate ozonide show characteristics similar to those of antioxidant protection against ozone itself. This observation provides additional support to the hypothesis of Menzel et al. (1;7;21) and Cortesi and Privett (8) that fatty acid ozonides are intermediates in the process of cell damage induced by ozone.

We have previously found that glutathione plays an important role in the protection of cells against ozone (11;17). However, the protective role of glutathione seemed not to be mediated by **i)** the glutathione peroxidase catalyzed detoxification of fatty acid hydroperoxides (11;12), **ii)** the formation of mixed disulfides (11) or **iii)**

regeneration of oxidized α-tocopherol (13). This suggested that glutathione may protect cells from ozone induced damage by acting as a scavenger of reactive intermediates, a hypothesis supported by the previous observation that intracellular vitamin C supplementation abolished part of the increased ozone sensitivity of glutathione depleted cells (13).

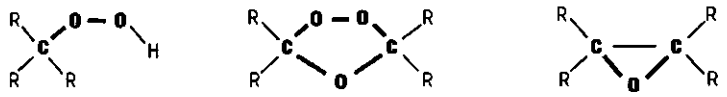
The results presented in this study clearly demonstrate the potential of glutathione to detoxify the ozonide of methyllinoleate, a model compound for likely intermediates in ozone toxicity. Perhaps surprisingly this detoxification could be increased considerably by the addition of rat liver glutathione S-transferase, to the preincubation mixture.

Methyl linoleate ozonide was shown to be a substrate for rat liver glutathione S-transferase with a Km_{app} of 0.80 mM. This means that the affinity of glutathione S-transferase for the ozonide model compound is of the same order of magnitude as its affinity for several other well-known substrates (22).

In theory, detoxification of the fatty acid ozonide by glutathione and glutathione S-transferase may proceed by a nucleophilic attack of glutathione on the ozonide ring structure, in analogy to the reaction between glutathione and epoxides or hydroperoxides. As depicted in figure 6 an ozonide has structural characteristics comparable to both of these classes of substrates. Reaction might thus take place at one of the carbon atoms of the ozonide ring structure, as is the case for epoxides (22;23), or on one of the oxygen atoms of the peroxy moiety present in the ozonide structure, as occurs with hydroperoxides (23;24).

Menzel et al. (7) reported the non-enzymatic oxidation of reduced glutathione by methyl ozonides to the oxidized disulfide form (GSSG), but their conclusion was based only on the disappearance of the reduced form of glutathione.

Preliminary results from our lab indicate that the glutathione S-transferase catalyzed detoxification of methyl linoleate ozonide



a. b. c.

Figure 6. A comparison of a) hydroperoxides, b) ozonides and c) epoxides. Reaction with glutathione might take place at the carbon atom of the ozonide as with epoxides or on one of the oxygen atoms of the peroxy moiety as in hydroperoxides.

results in formation of GSSG as well as of other glutathione-containing intermediates and/or final products. Elucidation of the exact mechanism of detoxification has to await the identification of these compounds.

In an intact cell system, detoxification of ozonized fatty acid membrane moieties by cytoplasmatic glutathione S-transferase may depend on migration of the polar ozonized group out of the inner membrane region and/or on the action of phospholipases which might recognize oxidized - thus perhaps also ozonized - membrane associated lipid chains, and cleave them, resulting in their release into the cytoplasm (25;26). In addition, the membrane bound microsomal glutathione S-transferase may play a role (27;28).

In summary, the glutathione S-transferase catalyzed detoxification of the potential ozonide intermediates, offers a completely new viewpoint on the role of glutathione in the protection of cells against ozone.

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PART III

SUMMARY AND CONCLUSIONS

CHAPTER 10

SUMMARY

Ozone and nitrogen dioxide are major toxic components of photochemical smog. They arise from the combustion of fossil fuels (traffic, industrial processes) and from solar radiation-catalyzed reactions in polluted atmospheres.

The morphological, physiological and biochemical effects of ozone and nitrogen dioxide on the respiratory system of man and experimental animals have been investigated over the last decades.
More recently the development of **i)** isolation and cell culture procedures for different types of lung cells and **ii)** model systems for *in vitro* exposure of cells to gaseous compounds, offers new possibilities to study the mechanism of toxic action of ozone and nitrogen dioxide. An advantage of the cell model is that changes in homogeneous cell populations can be studied, although it is recognized that effects found in isolated cell cultures always need *in vivo* validation.

The experiments described in this thesis were undertaken to further elucidate **i)** the mechanisms of action of ozone and nitrogen dioxide, as well as **ii)** the mechanisms of cellular protection against both gaseous compounds.
In vitro exposure of cells was achieved by using a system in which cells are grown on a thin teflon membrane and exposed by means of gas diffusion through this membrane.
Experiments were carried out using either cells from the A549 cell line or primary cultures of alveolar macrophages or alveolar type II pneumocytes, isolated from the lungs of control and in some cases (chapter 6a + b) from ozone or nitrogen dioxide exposed rats.

Part I of this thesis starts with a review of literature data on historical backgrounds, physical characteristics, concentrations encountered in the environment and toxic effects of ozone and nitrogen dioxide (chapter 1).
This is followed by a review on the current theories with regard to the mode of toxic action of ozone and nitrogen dioxide as well as to the mechanisms of cellular protection against these compounds (chapter 2).
Finally a review of in vitro exposure models is presented, including a description of the gas diffusion mediated exposure model applied in this thesis (chapter 3).

Part II of this thesis deals with the experiments carried out to obtain additional insight into **i)** the mode of toxic action of ozone and nitrogen dioxide and **ii)** the mechanisms providing protection against both gases in an intact cell system.
First experiments are described in which the mechanisms of toxic action of ozone and nitrogen dioxide were compared (chapter 4). In the in vitro exposure model applied ozone appeared to be 10 times more toxic than nitrogen dioxide. This difference is comparable with the difference in toxicity reported for in vivo exposures.
In addition it was demonstrated that the cellular antioxidant compounds, vitamin E (α-tocopherol), vitamin C (ascorbic acid) and glutathione, are all involved in the protection of cells against ozone or nitrogen dioxide.
The protection of α-tocopherol was shown to be dependent on its 6-hydroxyl group and not to be mediated by a structural stabilizing membrane effect of the compound arising from its strong physicochemical association with for example sensitive arachidonyl fatty acid residues. This could be concluded from the observations that **i)** α-tocopherol, at concentrations that provided optimal protection against the oxidative compounds, did not influence cell membrane fluidity and that **ii)** phytol and the methyl ether of α-tocopherol, two structural

α-tocopherol analogues, could not provide protection against ozone or nitrogen dioxide comparable to the α-tocopherol protection.
Furthermore chapter 4 presents in vitro data which clearly demonstrate that the reaction pathways involved in ozone and nitrogen dioxide induced cell damage must be different. This conclusion is based on the observations that **i)** vitamin C enrichment of cells provided significantly better protection against nitrogen dioxide than against an equally toxic amount of ozone, that **ii)** glutathione depletion increased the cellular sensitivity towards ozone to a greater extent than the sensitivity towards nitrogen dioxide and that **iii)** α-tocopherol dependent protection of the cells was accompanied by a significantly greater reduction in cellular α-tocopherol upon nitrogen dioxide than upon ozone exposure.
The observations referred to above are compatible with the hypothesis that ozone damage proceeds by the ionair mechanism for ozonide formation, whereas nitrogen dioxide induced cell damage is induced by a radical mediated lipid peroxidative pathway.
This hypothesis includes the involvement of oxidation of unsaturated membrane lipids in the mechanisms of ozone or nitrogen dioxide induced cell damage.

Evidence for the involvement of lipid oxidation in the mechanism of ozone or nitrogen dioxide induced cell damage is presented in the next chapter, (chapter 5), in which experiments are described that investigated the influence of polyunsaturated fatty acid (PUFA) supplementation on the sensitivity of cells towards ozone and nitrogen dioxide.
The results showed that cells enriched in their PUFA content demonstrate an increased sensitivity towards both ozone and nitrogen dioxide. It was also shown that this increased sensitivity was not caused by an increased membrane fluidity but really by the increased number of unsaturated fatty acids. Therefore these results clearly point to the involvement of lipid oxidation in the mechanism of action of both ozone and nitrogen dioxide.

In addition to the evidence for a difference in the mechanisms of toxic action of ozone and nitrogen dioxide observed in the in vitro experiments, evidence suggesting a difference in their toxicity was also obtained in in vivo experiments(chapter 6a + b). Exposure of rats to doses of both gaseous compounds that equally induced glucose-6-phosphate dehydrogenase activity in whole lung homogenates and in isolated alveolar macrophages and type II pneumocytes, resulted in a greater increase in the activity of glutathione peroxidase in cell material derived from ozone than from nitrogen dioxide exposed rats.

In addition it was shown that the increased activities of the enzymes of the glutathione peroxidase system, observed in lung homogenates of ozone or nitrogen dioxide exposed rats were caused by cell proliferation as well as by the increase of enzyme activities within individual cells.
In the literature these inductions have often been coupled to a glutathione dependent mechanism of cellular defense. To test this hypothesis alveolar macrophages and type II pneumocytes isolated from exposed rats were exposed to ozone or nitrogen dioxide in vitro. From these studies it appeared that cells derived from exposed animals revealed no increased resistance to the oxidative compounds as compared to the cells isolated from non-exposed animals. This in spite of a significantly increased glutathione peroxidase activity in the cells derived from exposed animals. Hence it can be concluded that an increased cellular glutathione peroxidase activity is not related to an increased cellular resistance towards ozone or nitrogen dioxide.

Data presented in the next chapter (chapter 7) demonstrated even more clearly that the glutathione dependent protection of cells against ozone is not mediated by the glutathione peroxidase catalyzed detoxification of fatty acid hydroperoxides. A549 cells showed a significantly increased sensitivity towards ozone upon depletion of their cellular glutathione content, which clearly points to a glutathione

dependent mechanism of cellular protection, although these cells do not contain a detectable glutathione peroxidase activity. This observation obviously excludes the glutathione peroxidase catalyzed detoxification of lipid hydroperoxides as a main mechanism for glutathione dependent cellular protection against ozone.

Additional results demonstrated the loss of glutathione from the cytoplasm of ozone exposed cells. This loss of glutathione from the cytoplasm of exposed cells was caused by leakage and/or active transport of glutathione out of exposed cells to the surrounding medium. The loss could not be ascribed to incorporation of a substantial amount of glutathione into mixed disulfides. This observation excludes a second hypothesis for the glutathione dependent protection of cells against ozone, viz. its incorporation into mixed disulfides, thus protecting cellular thiol groups from irreversible oxidation by ozone or its reactive intermediates.

The results presented in the next chapter (chapter 8), indicate that the increased ozone sensitivity of glutathione depleted cells is not caused by an impaired regeneration of α-tocopherol in these cells. This follows from the observations that **i)** glutathione depleted, ozone exposed cells did not contain decreased levels of α-tocopherol and that **ii)** vitamin E supplementation could not diminish the increased ozone sensitivity of the glutathione depleted cells.

So far all three hypotheses mentioned in the literature for a glutathione dependent cellular protection against ozone, had been excluded. From this, and from the observation that vitamin C supplementation abolished part of the increased ozone sensitivity of glutathione depleted cells, it is concluded that the most likely mechanism for a glutathione dependent protection of cells against ozone is provided by its action as a direct scavenger of reactive initial and/or intermediate species.

Experiments described in the final chapter of part II (chapter 9), actually demonstrate the ability of glutathione to detoxify a possible ozone intermediate; the ozonide derived from methyl linoleate. Methyl linoleate ozonide appeared to be toxic towards alveolar macrophages at concentrations between 10 to 100 μM, and showed characteristics with regard to cellular antioxidant protection that were similar to those for ozone itself.

In addition the detoxification of methyl linoleate ozonide by glutathione appeared to be even more pronounced when catalyzed by glutathione S-transferase.

Hence the glutathione S-transferase catalyzed detoxification of fatty acid ozonides provides a new point of view on the protective role of glutathione in ozone exposed cells.

Finally part III of this thesis summarizes all data and conclusions presented, both in english (chapter 10)and dutch (chapter 11).

CHAPTER 11

SAMENVATTING

Ozon en stikstof dioxide vormen belangrijke bestanddelen van fotochemische luchtverontreiniging. Ze ontstaan bij de verbranding van fossiele brandstoffen in het verkeer en bij industriele processen, en bij de door zonlicht gekatalyseerde reacties in de vervuilde atmosfeer.

In de afgelopen decennia zijn de morfologische, fysiologische en biochemische effecten van ozon en stikstof dioxide op de luchtwegen van de mens en van proefdieren uitvoerig bestudeerd.
De recente ontwikkeling van **i)** procedures voor het in cultuur brengen van verschillende typen longcellen en van **ii)** model systemen waarmee cellen *in vitro* aan gasvormige verbindingen kunnen worden blootgesteld, biedt nieuwe mogelijkheden voor de bestudering van met name het schademechanisme van ozon en stikstof dioxide. Een voordeel van het gebruik van celcultures is dat veranderingen in homogene populaties bestudeerd kunnen worden, alhoewel effecten, waargenomen in geïsoleerde celcultures, toch altijd extrapolatie naar de *in vivo* situatie behoeven.
De experimenten die in dit proefschrift worden beschreven waren speciaal gericht op **i)** het mechanisme via welk ozon en stikstof dioxide in een intact cellulair systeem schade aanrichten, en ook op **ii)** de mechanismen waarmee de cel zich tegen de schadelijke werking van ozon en stikstof dioxide kan beschermen.
Hierbij werd gebruik gemaakt van een *in vitro* expositie model waarin de cellen worden gekweekt op een dunne teflon membraan en vervolgens blootgesteld via de diffusie van de gasvormige verbinding door dit membraan. Voor de experimenten werd gebruik gemaakt van cellen van de

A549 cellijn, of van primaire culturen van alveolaire macrofagen of alveolaire type II cellen, geisoleerd uit de longen van controle ratten en - in een aantal gevallen - van aan ozon of stikstof dioxide blootgestelde ratten (hoofdstuk 6a + b).

Deel I van dit proefschrift begint met een overzicht van literatuurgegevens betreffende de historische achtergronden, de fysische eigenschappen, het voorkomen in de atmosfeer en de belangrijkste toxische effecten van ozon en stikstof dioxide (hoofdstuk 1).
Dit wordt gevolgd door een overzicht van de thans gangbare theorieën over de wijze waarop ozon en stikstof dioxide schade teweeg zouden kunnen brengen, als ook over de mechanismen die voor bescherming van de cel tegen deze oxiderende gassen in aanmerking zouden kunnen komen (hoofdstuk 2).
Deel I wordt besloten met een overzicht van gangbare in vitro expositie systemen en een beschrijving van het gasdiffusie expositie model, dat gebruikt werd voor de in dit proefschrift beschreven experimenten (hoofdstuk 3).

Deel II van dit proefschrift beschrijft in een 6-tal hoofdstukken de resultaten van het onderzoek.
Allereerst worden experimenten beschreven waarin de schade mechanismen van ozon en stikstof dioxide werden vergeleken (hoofdstuk 4). In het gebruikte in vitro expositie model bleek ozon 10 keer toxischer te zijn dan stikstof dioxide. Dit verschil is vergelijkbaar met het verschil in toxiciteit dat bij in vivo studies wordt waargenomen.
Ook werd aangetoond dat de cellulaire antioxidantia, vitamine E (α - tocoferol), vitamine C (ascorbine zuur) en glutathion, allen betrokken kunnen zijn bij de cellulaire afweer tegen ozon en stikstof dioxide. De bescherming door α-tocoferol bleek afhankelijk van de 6-hydroxyl groep binnen het molecuul. De bescherming wordt niet bewerkstelligd via een structureel stabilizerend membraan effect dat veroorzaakt zou kunnen worden door de sterke fysisch-chemische associatie tussen de

apolaire staart van het vitamine E molecuul en bijvoorbeeld arachidonzuur ketens in de membraan fosfolipiden. Een en ander kon worden geconcludeerd uit de waarneming dat **i)** α-tocoferol concentraties die optimale bescherming boden tegen ozon en stikstof dioxide, geen invloed hadden op de membraan vloeibaarheid en dat **ii)** fytol en de methyl ether van α-tocoferol, beiden structurele analogen van α-tocoferol, geen bescherming boden tegen ozon of stikstof dioxide, vergelijkbaar met de α-tocoferol bescherming.

Hoofdstuk 4 bevat eveneens gegevens waaruit duidelijk blijkt dat de reactiewegen via welke ozon dan wel stikstof dioxide schade teweeg brengen, verschillend zijn. Deze conclusie komt voort uit de volgende resultaten : **i)** vitamine C verrijking van de cellen bood significant betere bescherming tegen stikstof dioxide dan tegen ozon, **ii)** verlaging van het cellulaire glutathion gehalte vergrootte de ozon gevoeligheid van de cellen in sterkere mate dan hun stikstof dioxide gevoeligheid en **iii)** de vitamine E afhankelijke bescherming ging bij stikstof dioxide blootstelling gepaard met een significant grotere reductie van het cellulaire α-tocoferol gehalte, dan bij ozon blootstelling werd waargenomen.

Bovengenoemde resultaten zijn in overeenstemming met de hypothese dat ozon schade ontstaat via het ionaire mechanisme waarbij ozonides worden gevormd, terwijl stikstof dioxide schade zou ontstaan via het radicaal mechanisme voor vetzuur peroxidatie.

Een dergelijke hypothese gaat er wel vanuit dat ozon en stikstof dioxide schade ontstaat via een mechanisme waarin oxidatie van onverzadigde membraanvetzuren een rol speelt.

Een aanwijzing voor de betrokkenheid van vetzuur oxidatie in het reactie mechanisme van beide oxiderende verbindingen volgt uit de resultaten beschreven in het volgende hoofdstuk (hoofdstuk 5), dat experimenten beschrijft waarin de invloed werd bestudeerd van een verrijking van de cellen met meervoudig onverzadigde vetzuren (PUFA's) op hun gevoeligheid voor ozon en stikstof dioxide.

De resultaten van dit onderzoek toonden aan dat PUFA-verrijkte cellen een verhoogde gevoeligheid bezitten ten opzichte van beide oxiderende gassen. Bovendien werd aangetoond dat deze verhoogde gevoeligheid niet voortkomt uit de verhoogde membraan vloeibaarheid van de PUFA-verrijkte cellen, maar wel degelijk het gevolg is van een verhoogd aantal onverzadigde vetzuren in deze cellen. Derhalve duiden deze resultaten op de betrokkenheid van vetzuur oxidatie bij het schade mechanisme van ozon en stikstof dioxide.

Behalve de in vitro resultaten die duiden op een verschil in werkingsmechanisme tussen ozon en stikstof dioxide (hoofdstuk 4) worden in dit proefschrift ook resultaten beschreven die duiden op een verschil in in vivo toxiciteit (hoofdstuk 6a + b). Het bleek namelijk, na expositie van ratten aan concentraties ozon en stikstof dioxide die vergelijkbare inducties gaven van de glucose-6-fosfaat dehydrogenase activiteit (in zowel long homogenaat als in geisoleerde alveolaire macrofagen en type II pneumocyten), dat ozon een sterkere inductie veroorzaakte van de glutathion peroxidase activiteit dan stikstof dioxide.
Voorts werd aangetoond dat de verhoogde activiteit van de enzymen van het glutathion peroxidase systeem, waargenomen in long homogenaten van aan ozon of stikstof dioxide blootgestelde ratten, veroorzaakt wordt door zowel celproliferatie als door een verhoogde enzym activiteit in individuele longcellen.
In de literatuur worden deze inducties vaak gekoppeld aan een glutathion afhankelijk beschermingsmechanisme tegen de oxiderende gassen. Om deze hypothese te toetsen werden alveolaire macrofagen en type II pneumocyten, geisoleerd uit blootgestelde ratten of uit controle dieren, in vitro blootgesteld aan ozon of stikstof dioxide. De resultaten van deze experimenten laten zien dat de cellen afkomstig van blootgestelde dieren geen verhoogde resistentie tegen de oxiderende verbindingen bezitten. Dit, terwijl de cellen uit blootgestelde ratten wel een significant hogere glutathion peroxidase activiteit

bezitten, vergeleken met cellen afkomstig van controle dieren. Hieruit volgt dat een verhoogde glutathion peroxidase activiteit niet gekoppeld is aan een verhoogde cellulaire weerstand tegen ozon of stikstof dioxide.

Gegevens uit het volgende hoofdstuk (hoofdstuk 7) bevestigen, dat de glutathion afhankelijke bescherming van cellen tegen ozon niet verloopt via de glutathion peroxidase gekatalyseerde detoxificatie van vetzuur hydroperoxides. A549 cellen vertoonden namelijk na depletie van hun glutathion gehalte een verhoogde ozon gevoeligheid, hetgeen duidelijk wijst op een glutathion afhankelijk beschermingsmechanisme tegen ozon, hoewel deze cellen geen detecteerbare glutathion peroxidase activiteit bevatten. Dit sluit de glutathion peroxidase gekatalyseerde reactie uit als een belangrijk mechanisme voor glutathion afhankelijke cellulaire bescherming tegen ozon.
Verdere resultaten toonden aan dat aan ozon blootgestelde cellen een deel van hun glutathion verliezen. Dit verlies van glutathion uit het cytoplasma van aan ozon blootgestelde cellen werd veroorzaakt door een lekkage en/of een actief transport van glutathion uit de blootgestelde cellen naar het omringende medium. Het verlies kon niet worden toegeschreven aan de incorporatie van een aanzienlijke hoeveelheid glutathion in zogenoemde 'mixed disulfides'. Deze waarneming sluit een tweede hypothese voor de glutathion afhankelijke bescherming tegen ozon uit, namelijk degene die berust op de inbouw van glutathion in 'mixed disulfides', waardoor de cellulaire thiolgroepen kunnen worden beschermd tegen een irreversibele oxidatie door ozon of daarvan afkomstige intermediairen.

De in het volgende hoofdstuk (hoofdstuk 8) beschreven resultaten geven aan dat de verhoogde ozon gevoeligheid van glutathion gedepleteerde cellen ook niet veroorzaakt wordt door een aangetaste regeneratie van vitamine E. Een en ander volgt uit de volgende observaties : i) glutathion gedepleteerde, aan ozon blootgestelde cellen vertoonden

geen verlaagde α-tocoferol gehaltes en **ii)** vitamine E supplementatie van glutathion gedepleteerde cellen kon hun verhoogde ozon gevoeligheid niet opheffen.
In dit stadium van het onderzoek waren drie van de in de literatuur genoemde hypothesen voor een glutathion afhankelijk beschermingsmechanisme tegen ozon, afgevallen. Dit gegeven, in combinatie met het feit dat vitamine C supplementatie van glutathion gedepleteerde cellen hun verhoogde ozon gevoeligheid verminderde, maakt het waarschijnlijk dat de glutathion bescherming tegen ozon verloopt via een scavenger werking, waarbij glutathion initiele en/of intermediaire reactieproducten onschadelijk maakt.

In het laatste hoofdstuk van deel II (hoofdstuk 9) wordt het vermogen van glutathion om een theoretisch ozon intermediair te detoxificeren beschreven, te weten het ozonide van methyl-linoleaat. Methyl-linoleaat ozonide bleek toxisch voor alveolaire macrofagen bij concentraties van 10 tot 100 μM en vertoonde bovendien karakteristieken met betrekking tot de antioxidant bescherming die vergelijkbaar waren met die van de antioxidant bescherming tegen ozon zelf.
Voorts werd aangetoond dat de detoxificatie van methyl-linoleaat ozonide gekatalyseerd kan worden door glutathion S-transferase. Op basis van deze bevindingen kan worden gesteld dat de glutathion S-transferase afhankelijke detoxificatie van vetzuur ozonides een nieuw gezichtspunt oplevert met betrekking tot de beschermende rol van glutathion in aan ozon blootgestelde cellen.

Dit proefschrift wordt besloten met deel III, waarin een samenvatting van alle data en conclusies wordt gegeven, zowel in het Engels (hoofdstuk 10) als in het Nederlands (hoofdstuk 11).

CURRICULUM VITAE

Ivonne M.C.M. Rietjens werd geboren te Utrecht op 9 december 1958, en behaalde in 1977 het diploma Gymnasium β aan het Lyceum Augustinianum te Eindhoven. In datzelfde jaar werd begonnen met de studie moleculaire wetenschappen aan de Landbouwhogeschool waar zij in 1983 het ingenieursdiploma behaalde (met lof), met als hoofdvakken biochemie (prof.dr. C. Veeger) en toxicologie (prof.dr. J.H. Koeman) en als bijvak moleculaire genetica (prof.dr.ir. J.H. van der Veen).

Op 1 oktober 1983 startte zij - in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek - het promotieonderzoek bij de vakgroep Toxicologie, waarvan de resultaten zijn beschreven in dit proefschrift.

Sedert 1 oktober 1986 is zij werkzaam bij de afdeling Inhalatie toxicologie van het Rijks Instituut voor Volksgezondheid en Milieuhygiëne te Bilthoven.