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Oxidant Injury to the Alveolar Epithelium: Biochemical and Pharmacologic Studies

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Includes the Commentary of the Institute's Health Review Committee

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HEI HEALTH EFFECTS INSTITUTE

The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI studies all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate materials), and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 120 projects at institutions in North America and Europe.

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HEI Statement

Synopsis of Research Report Number 54

Mechanisms for Protecting Lung Epithelial Cells Against Oxidant Injury

BACKGROUND

Ozone and nitrogen dioxide are significant outdoor and indoor air pollutants that can cause lung damage. Both are termed oxidant gases because the oxygen atoms they contain react with a variety of lung components and produce injury. Depending on gas concentration and exercise intensity, people can experience altered breathing patterns after short periods of inhalation. Whether long-term inhalation causes or contributes to chronic lung disease is not known. Based on their reported adverse health effects, the Environmental Protection Agency has classified both gases as criteria air pollutants and has established National Ambient Air Quality Standards for them.

Cells lining the gas exchange region of the lungs (alveolar epithelial cells) are targets for injury caused by inhaling oxidant gases. These gases cause damage because they react with the cells and reactive oxygen molecules are generated. These molecules can produce an oxidant stress that overwhelms the cells' normal protective (antioxidant) mechanisms. This study, sponsored by the Health Effects Institute, examined oxidant injury to alveolar epithelial cells and tested whether supplementing the levels of antioxidants would modify the cells' resistance to damage.

APPROACH

Dr. Bruce Freeman and colleagues exposed cultures of rabbit alveolar epithelial cells to air or to 95% oxygen (hyperoxia) to study how these exposures affected the levels of antioxidants. Hyperoxia was used as a model of exposure to environmental oxidant gases because it also generates reactive oxygen molecules that produce lung injury. To test whether supplementing levels of antioxidants in the alveolar cells would protect against hyperoxic injury, the investigators packaged antioxidant enzymes and the antioxidant vitamin E in microscopic lipid membranes, called liposomes, and added them to the cells. They also instilled liposomes into rabbits' lungs, exposed the rabbits to air or 100% oxygen, and then evaluated changes in the levels of antioxidant enzymes in the lungs.

RESULTS AND IMPLICATIONS

The investigators successfully prepared liposomes that could deliver antioxidants to rabbits' lungs and to rabbit alveolar epithelial cells in culture; both procedures increased antioxidant levels. They reported that, although instilled liposomes were distributed to all rabbit lung lobes, antioxidant enzyme levels varied among lobes and with duration of exposure to air or hyperoxia. Using cultures of rat alveolar epithelial cells, the investigators mimicked an oxidant gas exposure by adding to the media specific enzymes that produced reactive oxygen molecules. Liposome supplementation increased the cells' resistance to this oxidant exposure.

Early in their study, the investigators observed that untreated rabbit alveolar epithelial cells in culture exhibited rapid decreases in levels of antioxidant enzymes when exposed to air or hyperoxia. Because enzyme levels were not maintained, the investigators decided that the responses of cultured cells exposed to oxidant gases with or without liposome supplementation would not accurately reflect the responses of alveolar cells in animals to similar exposures. Therefore, they curtailed many proposed experiments with hyperoxia and ozone designed to test the effects of antioxidant supplementation. Instead, they studied xanthine oxidase, an enzyme whose activity is suspected as an intracellular source of reactive oxygen molecules, and determined that its activity decreased with time in culture.

In summary, the investigators presented new observations concerning the responses of alveolar epithelial cells to oxidant stress and the potential benefits of supplementing the cellular levels of antioxidants with liposomes. By improving our understanding of how oxidant gases damage lung cells and thereby contribute to lung disease, such experiments provide information necessary for setting relevant air quality standards for these toxic air pollutants.

This Statement is a summary, prepared by the Health Effects Institute (HEI) and approved by the Board of Directors, of a research project sponsored by HEI from 1985 to 1989. This study was conducted by Dr. Bruce A. Freeman and associates at the University of Alabama at Birmingham. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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TABLE OF CONTENTS

HEI Research Report Number 54

Oxidant Injury to the Alveolar Epithelium: Biochemical and Pharmacologic Studies

Bruce A. Freeman, Peter C. Panus, Sadis Matalon, Barbara J. Buckley, and R. Randall Baker

I. HEI STATEMENT Health Effects Institute i

The Statement is a nontechnical summary, prepared by the HEI and approved by the Board of Directors, of the Investigators' Report and the Health Review Committee's Commentary.

II. INVESTIGATORS' REPORT Bruce A. Freeman et al. 1

When an HEI-funded study is completed, the investigators submit a final report. The Investigators' Report is first examined by three outside technical reviewers and a biostatistician. The Report and the reviewers' comments are then evaluated by members of the HEI Health Review Committee, who had no role in the selection or management of the project. During the review process, the investigators have an opportunity to exchange comments with the Review Committee, and, if necessary, revise their report.

Abstract 1
Introduction 1
Objectives 5
Methods 5
- Cell Culture 5
- Liposomal Preparation and Analysis 6
- In Vivo Liposomal Instillation 6
- Type II Cell Enzyme Assays 7
- Cytotoxicity Assessment 7
- Oxidant Exposure of Cells 7
- Type II Cell Surfactant Metabolism as Indicated by Incorporation of [^{3}H]Choline and [^{14}C]Palmitate into Phosphatidylcholine 8
- Statistical Methods 8

Results 8
- Type II Cell Phenotypic Characteristics and Antioxidant Enzyme Activities During in Vitro Culture in Air or Hyperoxia 8
- Type II Cell Cytotoxicity Markers and Their Response to Oxidant Stress 11
- Type II Cell Uptake of Liposomal Antioxidant Enzymes 13
- Type II Cell Uptake of Liposomal α-Tocopherol 15
- Protection of Oxidant-Stressed Type II Cells with Liposome-Delivered Antioxidants 16
- Pulmonary Delivery of Liposome-Entrapped Superoxide Dismutase and Catalase 18
- Type II Cell Xanthine Oxidase Is a Significant Source of Reactive Oxygen Species 19

Discussion 21
Summary 26
Acknowledgments 26
References 26
About the Authors 28
Publications Resulting from This Research 29
Abbreviations 29

III. COMMENTARY Health Review Committee 31

The Commentary on the Investigators' Report is prepared by the HEI Health Review Committee and staff. Its purpose is to place the study into a broader scientific context, to point out its strengths and limitations, and to discuss the remaining uncertainties and the implications of the findings for public health.

Introduction 31
Regulatory Background 31
Scientific Background 31
Justification for the Study 33
Specific Aims and Study Design 33
Technical Evaluation 34
- Attainment of Study Objectives 34
- Methods and Study Design 34
- Statistical Methods 35
- Results and Interpretation 35

Remaining Uncertainties and Implications for Future Research 36
Conclusions 37
References 38

IV. RELATED HEI PUBLICATIONS 40

INVESTIGATORS' REPORT

Oxidant Injury to the Alveolar Epithelium: Biochemical and Pharmacologic Studies

Bruce A. Freeman, Peter C. Panus, Sadis Matalon, Barbara J. Buckley, and R. Randall Baker

ABSTRACT

This multifaceted study involved a combined biochemical and cellular analysis of oxidant metabolism by a lung cell at risk from injury by endogenous and environmental oxidants, the pulmonary alveolar type II epithelial cell. Within the framework of this study, a method was developed for effectively delivering antioxidant enzymes and α-tocopherol to the intracellular compartment of alveolar epithelial cells.

Alveolar type II cells are key sources of pulmonary surfactant phospholipids and apoproteins and serve as progenitors of type I alveolar epithelium, thus playing an important role in the re-epithelialization of the lung alveolus after exposure to pulmonary oxidants. The type I and II pulmonary epithelium also play an essential collaborative role in maintaining the integrity of the air-blood barrier of the lung. Because of these critical properties of the alveolar epithelium and their recognized sensitivity to oxidant stress derived from diverse sources, such as activated inflammatory cells, hyperoxia, the environmental oxidants and nitrogen dioxide, and surgical procedures, such as cardiopulmonary bypass and lung transplantation, we endeavored to understand more about the oxidant metabolism and antioxidant pharmacology of these cells.

In our experiments, we made the observation that loss of differentiated oxidant generation and antioxidant properties of type II cells occurs very rapidly in vitro. For example, we observed a 50% to 75% reduction in the specific activities of type II cell superoxide dismutase, catalase, and glutathione peroxidase, all critical scavengers of cell superoxide and hydrogen peroxide and key enzymes in the attenuation of hydroxyl radical formation. Although the differentiated characteristics of the type II cell antioxidant defenses changed in vitro, they may have become more reflective of type I alveolar epithelial cells. The type I cell is the most vulnerable for oxidant damage in the alveolus because of its large surface area and the possibility of a reduced antioxidant capacity compared to type II alveolar epithelium.

In spite of this limitation, we were able to culture type II cells and study their adaptive and toxic responses to exogenously administered oxidant stress. We also observed that a significant source of self-generated oxidants in type II cells was the enzyme xanthine oxidase. Normal rates of oxidant production by this enzyme had an inhibitory effect on incorporation of biosynthetic precursors into surfactant phospholipids; these effects were eliminated by the xanthine oxidase inhibitor, allopurinol.

The ability of small (200 to 800 nm diameter) phospholipid membranes, termed liposomes, to deliver normally membrane-impermeable antioxidant enzymes or water-insoluble α-tocopherol to alveolar epithelial cells in vitro and in vivo was also investigated. The cell content of antioxidants could be specifically and rapidly increased, rendering cells and animals more resistant to oxidant stress. Alveolar epithelial cells were able to scavenge more effectively both intracellular and extracellularly derived oxidants. In summary, these experiments have developed a greater understanding of type II cell oxidant metabolism in vitro and in vivo and have yielded important new approaches to preventing lung injury during oxidant stress and for studying mechanisms of oxidant lung damage.

INTRODUCTION

Alterations in pulmonary anatomical and physiological parameters secondary to exposure to various gaseous oxidants are the focus of extensive toxicological investigation. Pulmonary damage induced by gaseous oxidants is, in part, due to the extensive surface area available to react with such gases. These oxidants are products of diverse sources, such as combustion engine emissions and oxygen (O_2) used for therapeutic purposes. Most previous pulmonary oxidant studies have focused on the biochemical mechanisms of toxicity of three of these gaseous oxidants: O_2, ozone (O_3), and nitrogen dioxide (NO_2). Although exposure to each of these agents can result in dose-dependent alterations in

This Investigators' Report is one part of the Health Effects Institute Research Report Number 54, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Bruce A. Freeman, Department of Anesthesiology, 956 Tinsley Harrison Tower, University Station, University of Alabama, Birmingham, AL 35294.

* A list of abbreviations appears at the end of this report for your reference.

pulmonary physiology and morphology, the detailed mechanisms of lung cellular injury rely upon the biochemical reactivity of each oxidant. Partially reduced oxygen species (PROS) may, in part, be involved in the biochemical mechanisms of damage induced by these gaseous oxidants.

In order to understand detailed toxicological mechanisms of oxidant gases and devise interventions to protect tissues at risk from oxidant injury, responses of specific cellular antioxidant protective mechanisms need to be well characterized and amenable to exogenous control. Thus, the investigation summarized herein of pulmonary type II alveolar epithelial responses to oxidant stress and the assessment of the potential use of liposomes as vectors for increasing lung antioxidant defenses was undertaken. As an introduction, responses of pulmonary tissue to oxidant stress, with an emphasis on alveolar epithelium, are discussed. We define oxidant stress as the diverse underlying biochemical events that result in the tissue pathology exhibited after it is exposed to a reactive oxidizing species. These biochemical events involve oxidation of thiol and aromatic amino acids, oxidation of unsaturated fatty acids, depletion of low molecular weight antioxidants (i.e., ascorbate, tocopherol, and glutathione), and oxidation of other key biomolecules such as enzyme cofactors and nucleic acid bases.

CROSS TOLERANCE

Preexposure of rats or mice to sublethal concentrations of NO_2, O_2, or O_3 results in "tolerance" to subsequent lethal concentrations of the same agent (Crapo and Tierney 1974; Douglas et al. 1977; Crapo et al. 1978; Jackson and Frank 1984). Preexposure to sublethal concentrations of one agent leads to "cross tolerance" for subsequent normally lethal concentrations of a different gaseous oxidant (Crapo and Tierney 1974; Crapo et al. 1978; Jackson and Frank 1984). Preexposure of rats to sublethal levels of 85% O_2 for five days resulted in subsequent cross tolerance to otherwise lethal levels of 75 parts per million (ppm) NO_2 (Crapo et al. 1978). Yet, preexposure of rats to sublethal NO_2 levels of 25 ppm for six hours/day for five days, did not result in cross tolerance to subsequent exposure at 100% O_2. Rats preexposed to 0.8 ppm O_3 for seven days were tolerant of subsequent exposure to 95% O_2 (Jackson and Frank 1984). Mice preexposed short term to 5 ppm O_3 for three hours became tolerant to subsequent normally lethal O_3 exposure two days later. However, these mice that were tolerant of O_3 were not tolerant to subsequent exposure to 98% O_2 (Douglas et al. 1977).

From these investigations, several important conclusions may be deduced. Induction of cross tolerance between two gaseous oxidants suggests that at least some common mechanisms of pulmonary damage and tissue adaptation may exist between those two agents. The different biochemical and cellular mechanisms resulting in cross tolerance between oxidant gases may also depend upon a multiplicity of additional factors including species-specific responses, oxidant concentrations, specific mechanisms of oxidant reaction with target molecules, and length of exposure.

CELL METABOLISM OF REACTIVE OXYGEN SPECIES

Partially reduced oxygen species, including cell superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) have been suggested as contributory mediators of the pulmonary toxicity induced by NO_2, O_2, and O_3. Although the electronic configuration of O_2 predisposes it to partially reduce to $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$, the mechanisms by which PROS mediate pulmonary toxicity induced by O_3 and NO_2 are less clearly defined. Extensive reviews of the cellular mechanisms of partially reduced oxygen formation are discussed elsewhere (Freeman and Crapo 1982; McCord 1983; Augst et al. 1985). Normally, 90% to 95% of all cellular oxygen consumption undergoes tetravalent reduction in mitochondria via cytochrome *c* oxidase, resulting in concomitant formation of water (H_2O) and adenosine triphosphate. Oxygen may also undergo univalent and divalent reduction to $O_2^{\bullet-}$ and H_2O_2, respectively. Further reactions of $O_2^{\bullet-}$ and H_2O_2 via redox cycling of transition metals results in $^{\bullet}OH$ formation. Antioxidant enzymes enzymatically reduce PROS to less reactive states. Superoxide dismutase (SOD), present in eukaryotic cells in both mitochondrial (manganese [Mn] SOD) and cytosolic (copper zinc [CuZn] SOD) forms, enzymatically dismutes $O_2^{\bullet-}$ to H_2O_2 and O_2. Hydrogen peroxide is reduced further to H_2O by peroxisomal catalase and other peroxidases, such as cytosolic glutathione peroxidase (GSH Px).

The above information demonstrates that partial reduction of oxygen results in direct PROS formation. The molecular nature of NO_2 and O_3 is different from O_2, with NO_2 being a radical and O_3 a zwitterion. Both NO_2 and O_3 react with olefins and proteins, with the products of these reactions being different (Menzel 1976; Mudd and Freeman 1977). These gases appear to attack both alkene bonds in olefins and intramolecular sulfhydryl and aromatic side chains within proteins. The reactions result in both peroxyl and alkyl radical products (Mudd and Freeman 1977; Prutz et al. 1985). The reaction of proteins and olefins with NO_2 also results in the formation of nitro-adduct derivatives (Menzel 1976; Prutz et al. 1985). At present, the direct reaction of NO_2 with olefins or proteins has not been demonstrated to generate directly partially reduced oxygen species, such as $O_2^{\bullet-}$ or H_2O_2. Alternatively, olefins have been shown to react with O_3 by a reaction mechanism termed

ozonolysis, which is not to be confused with lipid peroxidation; this results in the production of H_2O_2, with approximately 20% of the O_3 consumed in reactions yielding H_2O_2 (Mudd and Freeman 1977; Freeman et al. 1979). Similarly, O_3 has been shown to react with primary amines of low molecular weight compounds to produce H_2O_2 (Heath 1979). The reaction of O_3 with amines was conducted under basic conditions (pH 8.3); thus, its relevance to the in vivo situation is uncertain. Due to the relative availability of primary amines and olefins in vivo, the production of H_2O_2 by the reaction of O_3 with these compounds may play a role in pulmonary damage by O_3 (Pryor and Church 1991). Finally, the above investigations never clearly established that H_2O_2 was the direct product of O_3 reacting with olefins and amines. The $O_2^{\bullet-}$ may have been the direct product of the O_3 reaction, and the H_2O_2 detected may have represented a dismutation product of $O_2^{\bullet-}$.

RESPONSE OF PULMONARY ANTIOXIDANT DEFENSES TO OXIDANT STRESS

The involvement of PROS in pulmonary damage induced by oxidants may, in part, be inferred by examining changes in pulmonary antioxidant enzyme (AOE) levels in oxidant-tolerant animals. Rats that were made tolerant and cross tolerant to normally lethal levels of O_3 and O_2 by seven-day preexposure to sublethal concentrations of these same gases had enhanced pulmonary CuZn SOD, Mn SOD, catalase, and GSH Px activities (Jackson and Frank 1984; Freeman et al. 1986). Furthermore, it was demonstrated that augmentation of rat pulmonary SOD activity and resistance to a lethal 100% O_2 exposure both required at least three to five days of sublethal 85% O_2 exposure (Crapo and Tierney 1974). In contrast, rabbits that were exposed to 100% O_2 for 64 hours and then returned to normoxia for eight days were tolerant to 100% O_2 upon reexposure (Baker et al. 1989). These rabbits had no augmented pulmonary SOD or catalase activities and had no augmented pulmonary SOD, catalase, and GSH Px activities before and throughout a lethal 72-hour exposure to 100% O_2. Furthermore, mice exposed to three hours of sublethal O_3 were tolerant of subsequent normally lethal O_3, but not of normally lethal O_2 exposure two days later (Douglas et al. 1977). Augmented pulmonary SOD activities were not evident in these mice at the beginning of the lethal oxidant exposures. Catalase and GSH Px activities were not determined in these mice. The above investigations do not demonstrate a single unique AOE-dependent mechanism of tolerance to the various gaseous oxidants examined. Both the lack of augmented pulmonary AOE activities in O_2-tolerant rabbits, and the lack of O_2 cross tolerance and augmented pulmonary SOD activity in mice exposed to O_3 for a short term may be due to mechanisms of tolerance, such as shifts in cell populations, that are not dependent on antioxidant enzymes.

TISSUE SOURCES OF PARTIALLY REDUCED OXYGEN SPECIES

Observations of changes in whole lung AOE activities are not sufficient to demonstrate the participation of PROS in pulmonary damage due to elevated concentrations of O_3 and O_2. Such enzymatic changes may be due to cellular hyperplasia and hypertrophy secondary to diffuse lung injury and independent of biochemical mechanisms of tolerance in these animals. Measurement of elevated rates of PROS production in tissues during exposure to these oxidants would further demonstrate the involvement of PROS in pulmonary injury from O_3 and O_2. Possible loci of pulmonary PROS production during O_2 exposure include resident macrophages and neutrophils, as well as nonleukocytic sources. Determinations of the loci of intracellular PROS and mechanisms of tissue damage from PROS have been reviewed elsewhere (Freeman and Crapo 1982; Panus et al. 1988). These loci include electron transport systems, flavoproteins, and hemoproteins.

Recent investigations of oxidant-induced pulmonary damage have suggested a possibly important locus of intracellular, partially reduced oxygen formation, the oxidase form of xanthine dehydrogenase/xanthine oxidase (XDH/XO). Xanthine dehydrogenase/xanthine oxidase exists in two forms, both oxidizing a wide variety of purines and pteridine substrates. The XDH form reduces nicotinamide adenine dinucleotide (NAD+) to reduced nicotinamide adenine dinucleotide (NADH) and the XO form reduces molecular oxygen both univalently and divalently, forming $O_2^{\bullet-}$ and H_2O_2, respectively (McCord 1985). In situ conversion of the enzyme from the dehydrogenase to the oxidase form occurs as a result of disruption of tissue metabolic homeostasis by various mechanisms. Intramolecular sulfhydryl oxidation of purified XDH converts the enzyme to the oxidase form (Waud and Rajagopalan 1976). Reconversion of purified unproteolyzed oxidized XO back to XDH by reduction with sulfhydryl-reducing compounds has also been demonstrated (Waud and Rajagopalan 1976). Limited proteolysis converts XDH irreversibly to the XO form (Waud and Rajagopalan 1976; Engerson et al. 1987). Proteolytic conversion of purified XDH to XO has been demonstrated in both the presence and absence of sulfhydryl-reducing agents (Engerson et al. 1987). Whether limited proteolysis of XDH to XO follows sulfhydryl oxidation of XDH in situ has not been determined. Conversion of XDH to XO and subsequent $O_2^{\bullet-}$ and H_2O_2 production appear to play important roles in the pathophysiology of damage seen upon reoxygenation of ischemic tissues (Grisham et al. 1986; Parks et

al. 1988). Until recently, XDH/XO has been overlooked as an intracellular locus of partially reduced oxygen formation, which could mediate pulmonary damage induced by other toxic agents, such as O_2 and O_3.

Recent investigations show that exposure of adult rats to 100% O_2 for 24 hours resulted in a parallel loss of pulmonary XO and XDH activities (Terada et al. 1988). The cellular sources of pulmonary XDH/XO activity and mechanisms responsible for the loss of this activity were not established. Possible mechanisms include XDH to XO conversion and subsequent autoinactivation by self-produced PROS (Lynch and Fridovich 1979), extracellular release of XDH/XO into the vasculature, and proteolytic degradation and inactivation of both XDH and XO. The significance of cellular XDH/XO in mediating pulmonary damage in rats was further demonstrated by depleting rats of all XDH/XO with a diet high in tungsten. These rats then were exposed to 100% O_2 for 48 hours. When lungs from these animals were subsequently excised and perfused, they demonstrated less edema than their XDH/XO-replete and O_2-exposed counterparts (Rodell et al. 1987). The lungs from the tungsten-treated animals were also less susceptible to enhanced edema due to coperfusion with neutrophil elastase. Other investigations have demonstrated that oxidized proteins undergo accelerated proteolytic degradation (Davies 1986). Thus, partially reduced oxygen formation may exacerbate proteolytically induced tissue damage. The above studies, in aggregate, suggest that XDH/XO may be an important mediator of oxidant-induced pulmonary injury.

LIPOSOME-MEDIATED DELIVERY OF ANTIOXIDANT ENZYMES

If PROS are mediators of O_2- and O_3-induced pulmonary pathology and lethality, then augmentation of pulmonary AOE activities in animals should protect against these oxidant exposures. Delivery of native superoxide dismutase and catalase to rats by intravenous or intratracheal routes did not protect the animals from subsequent O_2 exposure and lethality (Turrens et al. 1984; Padmanabhan et al. 1985). However, delivery of liposome-entrapped AOEs diminished the lethality of O_2 exposure. Liposome entrapment provided a transmembrane vector for the intracellular delivery of normally membrane-impermeable macromolecules (Panus and Freeman 1988; Panus et al. 1988). Reduction of O_2 toxicity in rats receiving intravenous injections of liposomes required both SOD and catalase entrapped within liposomes (Turrens et al. 1984). In contrast, similar O_2-exposed animals receiving intratracheal delivery of liposomes containing either SOD or catalase alone were protected from O_2 toxicity (Padmanabhan et al. 1985). The pharmacokinetics of pulmonary delivery of liposome-entrapped AOEs, the importance of liposome membrane composition, and effects of AOE-entrapped liposomes on inflammatory responses are discussed elsewhere (Turrens et al. 1984; Padmanabhan et al. 1985). These results suggest that intracellular PROS production and damage may be a significant factor in O_2-induced pulmonary injury and subsequent lethality. Whether liposomal delivery of AOE also protects against O_3-induced lethality is, at present, undetermined. However, if O_3-induced pulmonary toxicity is mediated via PROS, then liposomal supplementation of AOEs should provide protection against O_3 injury. This issue was an experimental goal of the original study design but was outside the scope of final endeavors. Because liposomes are considered incapable of crossing the pulmonary basement membrane, the above investigations demonstrate differences in the AOE requirements of cells on opposite sides of the air-blood barrier. Thus, when developing an antioxidant pharmacologic regimen, specific AOE requirements of target cells on the intravascular and alveolar sides of the air-blood barrier must be clarified.

OXIDANT STRESS AND TYPE II CELL RESPONSES

A common anatomic change in the lung after chronic exposure to NO_2, O_2, or O_3 is the proliferation and hypertrophy of type II cells (Crapo et al. 1984; Evans 1984). Type II cell functions include surfactant metabolism, electrolyte transport, and re-epithelialization of the alveolar basement membrane by transformation into type I epithelium. As such, changes in type II cell number, function, or morphology could result in overall changes in pulmonary metabolism and function. Investigations have been conducted into whether oxidant exposure augments individual type II cell AOE activities, and whether subsequent tolerance is due in part to hypertrophy and hyperplasia of these cells. Isolated type II cells from rats, made tolerant by preexposure to 85% O_2 for seven days, showed augmented protein to DNA ratios and augmented cellular AOE activities when normalized for cell number or DNA (Freeman et al. 1986). Activities of CuZn and Mn SOD, catalase, and glutathione peroxidase increased in cells isolated from tolerant animals. Cellular hypertrophy was also assessed to determine whether increased cellular volume alone accounted for the augmented cellular AOE activities. When normalized for protein or cell volume, isolated oxygen-adapted type II cells showed only elevated CuZn and Mn SOD activities. Furthermore, type II cells in oxygen-tolerant rats contributed a greater percentage to the total lung AOE content than did type II cells in control rats. Thus, elevated pulmonary AOE activities in

O_2-tolerant rats are due both to type II cell hypertrophy and hyperplasia and to an increase in SOD concentration per unit of cell volume.

Alterations in type II cell AOE activities were also examined after exposure of rats to 80% O_2 for seven days (Haagsman et al. 1985). In type II cells isolated from these oxygen-adapted rats, only Mn SOD activity was elevated, with no change in CuZn SOD, catalase, or GSH Px activities. In this latter study, unlike the previous study (Freeman et al. 1986), antioxidant enzyme activities were normalized only for DNA. Ratios of cellular protein to DNA were not examined, and no determinations were made as to whether the change in Mn SOD activity was due to cell hypertrophy. Differences in results between these two type II cell studies from oxygen-adapted lungs may be due to variations in type II cell isolation methods utilized by the different investigators.

Type II cells isolated from rats exposed to 0.8 ppm O_3 for four days also have been examined for changes in cellular AOE activities (Rietjens et al. 1985). Isolated type II cells had enhanced GSH Px activity, even after accounting for O_3-induced cellular hypertrophy. Changes in cellular SOD and catalase activities and calculation of the fraction that type II cells contributed toward augmentation of whole lung AOE activities in these animals was not determined. In summary, changes in type II cell AOE activities can be due to enhanced intracellular activity, cell hypertrophy, and cell hyperplasia, which in O_2- and O_3-tolerant animals will be reflected by changes in whole lung AOE activities. Type II cells are important loci of pulmonary AOE activities and are important in the development of tolerance to O_2 and O_3 exposure.

PRODUCTION OF REACTIVE OXYGEN SPECIES IN TYPE II CELLS

Antioxidant enzyme activities in type II cells isolated from O_2- and O_3-tolerant animals suggest elevated rates of intracellular production of PROS in these cells. Several intramitochondrial and cytosolic sources produce $O_2^{\cdot-}$ and H_2O_2 during normoxic conditions and at enhanced rates in hyperoxia (Freeman and Crapo 1982). During exposure to O_2 and O_3, the XDH/XO locus may also be an important site of PROS production in type II cells. Ozone exposure of whole animals or isolated type II cells inhibits glycerolphosphate acyltransferase and choline phosphotransferase of the surfactant synthesis pathway, as well as the glucose-6-phosphate dehydrogenase necessary for maintaining cellular reduced nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione levels (DeLucia et al. 1972; Haagsman et al. 1985). Ozone-induced inactivation of these enzymes appears to be mediated via sulfhydryl oxidation of the proteins. Thus, loss of these enzyme activities in cells can serve as a possible marker of oxidant stress.

The present study was directed toward developing a better understanding of PROS metabolism by alveolar epithelial type II cells. This was addressed first by isolating cells and studying their endogenous antioxidant enzyme-specific activities during in vitro culture and after an oxidant stress consisting of 95% O_2 or after exposure to PROS generated enzymatically in tissue culture medium. Then, type II cell responses to oxidant stresses were studied after liposome-mediated augmentation of antioxidant defense.

OBJECTIVES

This project examined the ability of liposomes containing antioxidants to alter antioxidant defenses of the alveolar epithelium and modify the response of these cells to oxidant stress. The liposomes served as vectors for the transmembrane delivery of normally membrane-impermeable or aqueous-insoluble macromolecules. The cell and pulmonary distribution and fate of liposome-delivered molecules were quantified using fluorescence and enzymological techniques. The hypothesis guiding our proposed studies was that oxidant stress increases the intracellular rate of production of PROS. The efficient delivery of enzymatic antioxidant defenses to the cytosolic compartment and of hydrophobic antioxidants, such as α-tocopherol, to membranes would provide cells with an enhanced ability to survive periods of acute oxidant stress. In this study, oxidant stress was induced by exposing tissues to 95% O_2, 100% O_2, or reactive O_2 species generated enzymatically in tissue culture medium.

METHODS

CELL CULTURE

Alveolar type II cells were isolated and cultured from Sprague-Dawley rats. Lungs were digested via an elastase-trypsin proteolysis and subsequently minced and filtered, before centrifugation over a Percoll gradient. Cells suspended in the gradient were subsequently removed and diluted, centrifuged (200 × g, 10 minutes, 4°C), and incubated in rat IgG-precoated plates for one hour. Nonadherent cells were panned off and centrifuged in the method described above. Cell pellets then were resuspended in culture medium consisting of Ham's F12 and Dulbecco's modified Eagle medium (1:1) (GIBCO, Grand Island, NY), 2% (v/v) fetal

bovine serum (Hyclone, Logan, UT), 1% antibiotics/antimycotic (GIBCO), 0.1 mg/mL soybean trypsin inhibitor (Sigma, St. Louis, MO), 3 μg/mL insulin and transferrin, and 3 ng/mL selenous acid (Collaborative Research, Lexington, MA). Cells were plated on fibronectin precoated Costar 9.6 cm^2 dishes with a plating density of $4.4 \pm 2.6 \times 10^5$ cells/cm^2 (± SD) at 2 mL total volume/dish, and a purity of 84 ± 8% (± SD). Type II cell preparations were counted using a hemocytometer or Coulter Counter and purity was determined by phosphine-3R staining of lamellar bodies. Cell viability at plating was greater than 85%, as determined by Trypan blue dye exclusion. Cells were allowed to adhere for 36 to 39 hours under normoxic conditions (95% air, 5% CO_2). After the 36-hour adherence period, the cells were rinsed twice with fresh culture media and 2 mL of media was added. For biochemical analysis, adhered cells were rinsed twice with Hank's balanced salt solution (2 mL, 37°C) and sonicated in a lysing buffer (4°C) containing 50 mM potassium phosphate (pH 7.4), 0.1 mM ethylenediaminetetraacetic acid, 0.1% 3-[(3-cholamidopropyl)dimethylamino]-propanesulfonate (CHAPS), 1.0 mM phenylmethylsulfonyl fluoride, 0.5 mg/L leupeptin, and 10 mM dithiothreitol. Cellular homogenates then were frozen rapidly in −86°C isopentane and stored at −86°C until analysis.

For comparative purposes, and when larger cell numbers were required for experiments, alveolar type II cells also were isolated from rabbit lungs using standard procedures by elastase-trypsin digestion of lungs. This was followed by mincing and filtering the digested lung and centrifuging it on a Percoll gradient. Cells suspended in the gradient were centrifuged, resuspended, then incubated in rabbit IgG pretreated flasks to remove remaining contaminating lymphocytes and monocytes. Nonadherent cells were panned off the flask, pelleted in the method described above, resuspended in culture medium containing Dulbecco's modified minimum essential medium and Ham's F12 nutrient mixture (1:1, GIBCO), 2% fetal bovine serum (Hyclone), 1% antibiotic-antimycotic (GIBCO), 0.1 mg/mL soybean trypsin inhibitor (Sigma), 3 μg/mL insulin and transferrin, and 3 ng/mL selenous acid (Collaborative Research), and then plated onto Costar 9.6-cm^2 dishes pretreated with fibronectin. Plating density of cells was $1.01 \pm 0.57 \times 10^6/cm^2$, of which 83% ± 3% (mean ± SD) were type II cells. Cells were allowed to adhere for 36 to 39 hours before rinsing and addition of fresh culture medium.

LIPOSOMAL PREPARATION AND ANALYSIS

Small unilamellar liposomes were prepared by reverse-phase evaporation having cationic (dipalmitoylphosphatidylcholine [DPPC], cholesterol, stearylamine, 14:7:4 mol/mol), anionic (phosphatidylserine, cholesterol, 3:1 mol/mol), or neutral (dipalmitoylphosphatidylcholine, cholesterol, 3:1 mol/mol) charges. Cholesteryl oleate (oleate-1-^{14}C) incorporated into the lipid bilayer during liposome production was used to quantify radiochemically liposome adherence and uptake by cells. For entrapment of antioxidants, bovine CuZn superoxide dismutase (Grunenthal, GmBh), succinoylated bovine catalase (Cooper Biomedical) and D,L-α-tocopherol (Eastman) were included at 5 mg/mL protein in 10 mM potassium phosphate for SOD and catalase, and as an ethanol-dissolved solution for α-tocopherol. In some cases, large unilamellar liposomes were prepared by sonication (Szoka and Papahadjopoulos 1980). For fluorescence distribution studies, superoxide dismutase was labeled with fluorescein isothiocyanate. α-Tocopherol was quantified fluorometrically.

IN VIVO LIPOSOMAL INSTILLATION

Cationic liposomes consisting of dipalmitoylphosphatidylcholine:cholesterol:stearylamine (14:7:4, mol/mol) were prepared with either bovine CuZn SOD or succinoylated bovine catalase entrapped. Liposomes were resuspended in phosphate-buffered saline, pH 7.4, at a concentration of 20 μmol phospholipid/mL. To follow in situ liposome distribution, fluorescein isothiocyanate was covalently conjugated to CuZn SOD. Unconjugated fluorescein isothiocyanate was separated from fluorescently labeled enzyme by gel exclusion chromatography on Sephadex G-25. For liposome distribution studies, 10% of the liposome-entrapped protein was fluorescently labeled, the remainder being native protein. For liposome instillation, rabbits were anesthetized with 10 mg/kg ketamine injection via a marginal ear vein and maintained with 1.5% halothane in 4 L/min 100% O_2. Rabbits were placed on a surgical table which rotated through 360° on its longitudinal axis, with the head of the surgical table elevated 5°. Liposomes were instilled into lungs via a 22 gauge 2-inch catheter inserted percutaneously through the cricothyroid membrane. During the instillation, the surgical table was rotated slowly to enhance uniform liposome delivery to all lung fields. Approximately 20 μmol of liposome phospholipid containing 2,500 U CuZn SOD and 1,000 U catalase activity in a total volume of 10 mL phosphate-buffered saline was instilled into each rabbit. The instilled volume was approximately 10% of total lung capacity with all rabbits surviving liposome instillation and breathing normally shortly after the procedure. At various times after liposome instillation, rabbits were killed by sodium pentobarbital injection (65 mg/kg) into a marginal ear vein. The trachea was cannulated and lungs were isolated and perfused via the pulmonary artery with cold phosphate-buffered saline until the perfusate was clear. The

lungs and trachea were removed, weighed, and lavaged repeatedly with 30 mL phosphate-buffered saline until a total volume of 400 mL was delivered. The resultant bronchoalveolar lavage fluid was centrifuged at 200 × *g* for 6 minutes to remove cells and cellular debris. Lungs were weighed and each lobe separately minced and homogenized in 10 mL/g tissue, cold 50 mM potassium phosphate, and 0.1 mM EDTA, pH 7.8, and centrifuged at 200 × *g* for 10 minutes to remove cellular debris. Supernatants were stored at −70°C for subsequent analysis of SOD and catalase activity.

TYPE II CELL ENZYME ASSAYS

Lactate dehydrogenase (LDH) activity and DNA from cell-free supernatants and adherent cell lysates were determined spectrophotometrically (Haagsman et al. 1985) and fluorometrically (Fiszer-Szafarz et al. 1981), respectively. Cell-associated catalase and GSH Px activities were determined spectrophotometrically (Beutler 1975; Freeman et al. 1983). Superoxide dismutase activity was determined by a "burst" assay that utilized inhibition of xanthine- and XO–generated O_2 via assessment of cytochrome *c* reduction at pH 10.2. We found this assay to be more sensitive than the standard pH 7.8 cytochrome *c* reduction SOD assay (Kirby and Fridovich 1982). Cell-associated protein levels were determined by the Bradford dye binding technique (Bradford 1976) (reagent from Pierce, Rockford, IL) using bovine serum albumin as a protein standard. Cellular XDH/XO activity was determined via a spectrofluorometric assay that followed the oxidation of pterin to the fluorescent product isoxanthopterin ($\lambda_{excitation}$ = 345 nm, $\lambda_{emission}$ = 390 nm) (Beckman et al. 1989). Methylene blue was substituted for NAD+ as an electron acceptor when measuring total XDH/XO activity. Allopurinol (10 μM) was utilized to demonstrate that increases in fluorescence were due to XDH/XO activity. Individual sample quenching was adjusted for by adding known amounts of isoxanthopterin. The percentage of XO activity (% XO) was determined by dividing the XO activity by the total cellular XDH plus XO activity and multiplying by 100. One unit of enzymatic activity was defined as converting 1 μmol of pterin to isoxanthopterin per minute.

CYTOTOXICITY ASSESSMENT

Cytotoxicity was evaluated by a number of approaches as we learned about the strong potential for artifacts inherent in standard cytotoxicity assays during the course of our investigations. The release of intracellular contents from cell monolayers into culture medium was the basis for cytotoxicity assays. The release of type II cell LDH, DNA, ^{51}Cr prelabel, and ^{14}C-adenine prelabel to culture medium was assessed (Andreoli et al. 1985). Calculation of the percentage of radiolabel release from cell monolayers was based on the measurement of total radiolabel content of control cell lysates induced by 30 minutes of incubation of cells with 0.1% Triton-X-100 in culture medium. Our observation of cultured type II cells exposed to hyperoxia showed that both cellular and purified LDH is catalytically inactivated by various PROS. Cellular release of LDH activity is commonly utilized as a marker of cellular damage, in vitro. Thus, experiments on cell-free LDH (Sigma) were initiated to determine whether the enzyme was inactivated when exposed to PROS. Inactivation studies of LDH were conducted in M199 (pH 7.4, 25°C) with added $NaHCO_3$ and L-glutamine, and without phenol red (GIBCO). Xanthine (500 μM) and XO were added. Additional XO was added every 30 to 60 minutes to maintain an activity of 3.1 ± 1.3 mU/mL (mean ± SD). At no time was the xanthine rate limiting. Xanthine oxidase activity was determined by removing aliquots from the LDH reaction flask and measuring continuous changes in absorbance at 295 nm (Waud and Rajagopalan 1976). When XO activity fell below the required level, an aliquot from a stock XO solution was added to the LDH reaction and activity was redetermined. A final concentration of 82.5-μM H_2O_2 was added to separate LDH reactions at 0, 2, and 4 hours. At specific times aliquots were removed and LDH activity was determined in the method described above.

OXIDANT EXPOSURE OF CELLS

After plating freshly isolated cells for 36 to 39 hours, nonadherent cells were removed from culture dishes by panning, and fresh culture medium (2 mL/dish) was added. For hyperoxic exposure, dishes were incubated at 37°C for various periods of time and flushed at a minimum rate of 0.5 L/min with either a prehumidified and preheated (37°C) mixture of 95% air and 5% CO_2 (normoxia), or a mixture of 95% O_2 and 5% CO_2 (hyperoxia). Oxygen concentration in solution rose to at least 650 mm Hg within two hours, as determined by removing the culture medium and quantifying the amount of dissolved O_2 in a blood gas analyzer. At the termination of each experiment, the supernatant was pipetted from the dish, centrifuged (450 × *g*, 10 minutes, 4°C) to remove nonadherent cells and cellular debris, and the supernatant was stored (−20°C) for further analysis. Adherent cells were rinsed twice with Hank's balanced salt solution. For exposure to enzymatically generated oxidants, cells were maintained in serum-free medium, and 0 to 20 mU/mL XO plus 100 μM xanthine was added to the culture medium to generate $O_2^{\cdot-}$, H_2O_2, and •OH. To generate H_2O_2, 0 to 200 mU/mL glucose oxidase plus 11.2 mm glucose was added to the culture medium.

TYPE II CELL SURFACTANT METABOLISM AS INDICATED BY INCORPORATION OF [^{3}H]CHOLINE AND [^{14}C]PALMITATE INTO PHOSPHATIDYLCHOLINE

Trace amounts (2 μCi/mL) of [methyl-^{3}H]choline chloride (80 Ci/mmol) (DuPont) and [1-^{14}C]palmitic acid (58 mCi/mmol) (DuPont) were added to 2 × 10^6 type II cells/mL in Eagle's minimum essential medium. Palmitic acid was complexed to bovine serum albumin by dissolving it in 0.1 N potassium hydroxide (KOH) followed by neutralization with 0.1 N hydrochloric acid. Choline chloride (100 μM) was added to increase the substrate pool for choline. The cells were incubated in polypropylene culture tubes at 37°C in a gas mixture of 95% air and 5% CO_2. The substrate pools were allowed to equilibrate for 60 minutes before the first sample collection. After this period of time (t = 0) and two subsequent 30-minute intervals (t = 30 and t = 60), precursor incorporation was stopped by the addition of 2 mL of 4°C phosphate-buffered saline. Cells were centrifuged at 200 × *g* at 4°C for 60 minutes, and lipids were extracted from the cell pellets using chloroform and methanol (1:2). The organic layer was collected and dried. The lipids were resuspended in chloroform and separated by thin-layer chromatography using a solvent system composed of chloroform:methanol:2-propanol:water:triethylamine (3:9:25:7:25). The lipids were made visual with rhodamine-6B (Sigma), a fluorescent lipophilic stain. A trace amount of dipalmitoylphosphatidylcholine (Avanti Polar Lipids), was dissolved in chloroform and applied to the TLC plate as a standard. Each total phosphatidylcholine spot was scraped from the plate, placed into a scintillation vial containing 0.5 mL water and 10 mL Aquasol scintillation fluid, and counted by standard techniques.

STATISTICAL METHODS

All values are shown as the mean ± SEM, unless otherwise stated. Significance ($p<0.05$) was typically determined by analysis of variance, followed by a Duncan post hoc groupwise comparison on the dependent variable. Statistical analyses were done using "SAS System under PC DOS".

RESULTS

TYPE II CELL PHENOTYPIC CHARACTERISTICS AND ANTIOXIDANT ENZYME ACTIVITIES DURING IN VITRO CULTURE IN AIR OR HYPEROXIA

In order to understand the responses to oxidant stress of control type II cells and type II cells having liposome-mediated increases in antioxidant capacity, we studied key properties of these cells when maintained in primary culture in either normoxia or 95% O_2. In this series of experiments, which required large numbers of cells for biochemical analyses, type II cells were isolated from rabbits. Plating efficiency of type II cells on dishes pretreated with fibronectin (2.1 μg/cm^2) was approximately 20%, as determined by culture-associated DNA (Table 1). During the 39-

Table 1. Alterations in Cellular Characteristics of Freshly Isolated and Adherent Rabbit Type II Cells[a]

Measurement	Freshly Isolated Cells	*n*	Adherent Cells	*n*
Cellular (μg/cm^2)				
Protein	52 ± 6	2	4.8 ± 0.9	4
DNA	6.7 ± 0.1	2	1.3 ± 0.10	4
Protein/DNA	7.8 ± 1.0	2	3.8 ± 0.6	4
Enzyme activity (U/mg protein)				
SOD	28 ± 4	2	195 ± 47	4
Catalase	20 ± 11	2	57 ± 10	4
LDH	0.11 ± 0.0002	2	0.54 ± 0.23	4
Enzyme activity (U/mg DNA)				
SOD	213 ± 4	2	667 ± 83	4
Catalase	166 ± 105	2	202 ± 20	4
LDH	0.87 ± 0.10	2	1.7 ± 0.37	4

[a] Type II cell protein, DNA, and cellular enzymatic activities were measured in freshly isolated cells (plating at *t* = 0 hours) and cells adhered for 39 hours on 9.6-cm^2 fibronectin pretreated dishes, as described in the Methods section. The 39-hour adherence period was a component of the cell isolation and purification procedure. Cellular enzymatic activities, SOD, catalase, and LDH were normalized for both cellular protein and DNA due to the differential effects of culture on each of these denominators. The table represents the average of two separate experiments with initial values done with single dishes, and the 39-hour adherent cell values done with duplicate dishes. Each value represents the mean ± SEM. The values for freshly isolated cells represent what was originally plated on culture dishes.

hour adherence period, there was a greater loss of monolayer-associated protein than of DNA. The preferential loss in protein was evident from the decreased ratio of cell protein to DNA.

Viability and purity of type II cells after 24 hours in culture were assessed by exclusion of trypan blue and lamellar body staining, respectively. With these methods, more than 90% of the cells were found to exclude trypan blue and more than 85% of the cells were observed to contain lamellar bodies. Cell morphology, examined by electron microscopy, demonstrated the presence of numerous lamellar bodies, apical microvilli, and intercellular tight junctions in cells cultured for up to four days.

Comparison of cellular SOD, catalase, and LDH activities in freshly isolated and adherent cells demonstrated an apparent increase in enzyme activities in the adhered cells (Table 1). Increased cell-associated enzyme activities during the initial culture may reflect cellular responses to culture conditions. Alternatively, measurements of protein or DNA in freshly isolated cell suspensions may include nonviable cells that have lost cellular contents, thus artifactually reducing apparent enzyme-specific activities in freshly isolated cells.

After cell isolation and plating, the cultures were subjected to 0 to 96 hours of either normoxia or hyperoxia. Exposure of cells to normoxia for 96 hours resulted in a fivefold increase in protein content (Figure 1), whereas DNA only doubled (data not shown). The increased cell protein accounted for the augmented ratios of protein to DNA (Figure 1). During this same interval of normoxia, the activities of several different cellular antioxidant enzymes (catalase, GSH Px, and SOD) decreased (Figure 2). No distinction between Mn and CuZn SOD was made, due to limitations of cell mass and the assay system employed. In contrast to antioxidant enzymes, cellular LDH activity increased during the same time period (Figure 2).

Hyperoxic exposure of type II cells resulted in several phenotypic changes not observed in normoxic controls. The protein and DNA content of type II cell cultures exposed to hyperoxia for 96 hours was 32% (Figure 1) and 69% (data not shown), respectively, for similarly exposed normoxic controls. Hyperoxic exposure seemed to have different effects on cellular protein and DNA, resulting in a 53% decrease in the protein to DNA ratio when compared with results from normoxic controls (Figure 1). Trends for the loss of cellular antioxidant enzymes during hyperoxia paralleled normoxic controls (Figure 2). During 96 hours of hyperoxia, the loss of cell-associated catalase and GSH Px activities paralleled enzyme decreases in normoxia-exposed cultures. The loss of cellular SOD activity during hyperoxia, however, was less than that for normoxic controls (Figure 2). Decreased cellular LDH activity in hyperoxic cultures may have been due to inhibition of protein synthesis, enhanced rates of protein degradation, enhanced release of LDH into the supernatant, hyperoxia-induced inactivation of LDH, or a combination of these factors. Due to the different effects of hyperoxia on cellular protein and DNA content, cellular antioxidant enzyme-specific activities for oxygen and air-exposed cultures were also normalized for cellular DNA (Figure 3). Antioxidant enzyme activities in both exposure groups decreased in a fashion similar to that shown in Figure 2. However, the mean activities of cellular catalase and GSH Px in the hyperoxic cells were depressed, compared with air controls. Furthermore, the augmented cellular SOD activity in the hyperoxic-exposed group was not apparent. Finally, normalization of cellular LDH activity for DNA resulted in a further decrease in enzyme-specific activity in the hyperoxic-exposed cultures than in normoxic controls (Figure 3). Oxygen toxicity to type II cell cultures was demonstrated by the increased release of cellular LDH, DNA, and preincorporated 8-[^{14}C]-adenine (Figure 4). Extracellular release of all three markers also occurred during normoxia, but to a lesser extent.

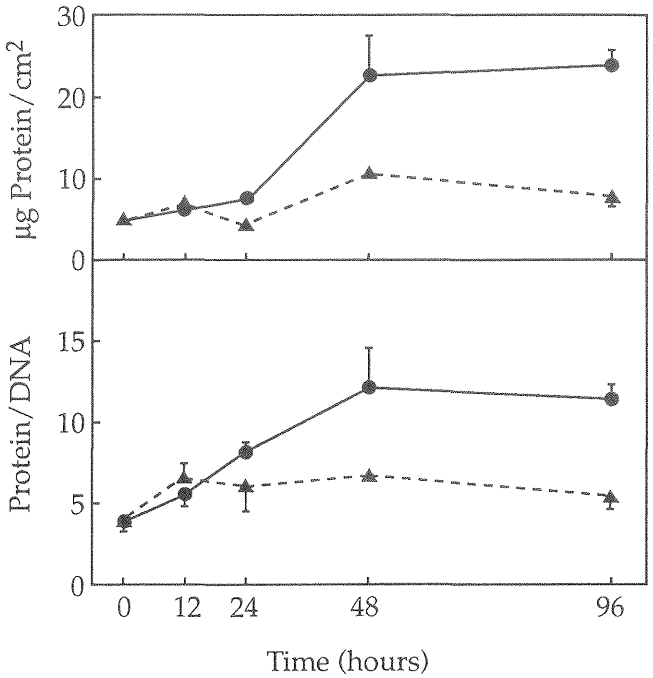

Figure 1. Protein content and protein:DNA ratios in cultured rabbit type II cells after 96 hours under normoxic (95% air, 5% CO_2) (●) and hyperoxic (95% O_2, 5% CO_2) (▲) conditions. Cells were allowed to adhere for 39 hours before initiation of the experiment. The top figure shows culture-associated protein (μg) normalized for culture dish surface area (cm^2). The bottom figure demonstrates the change in cellular protein to DNA ratios (μg/μg). Each time point represents the mean and SEM of at least two separate measurements. Each graph is the sum of two separate experiments. At several time points, the increment of variation was so small that it was not demonstrably separable from the mean.

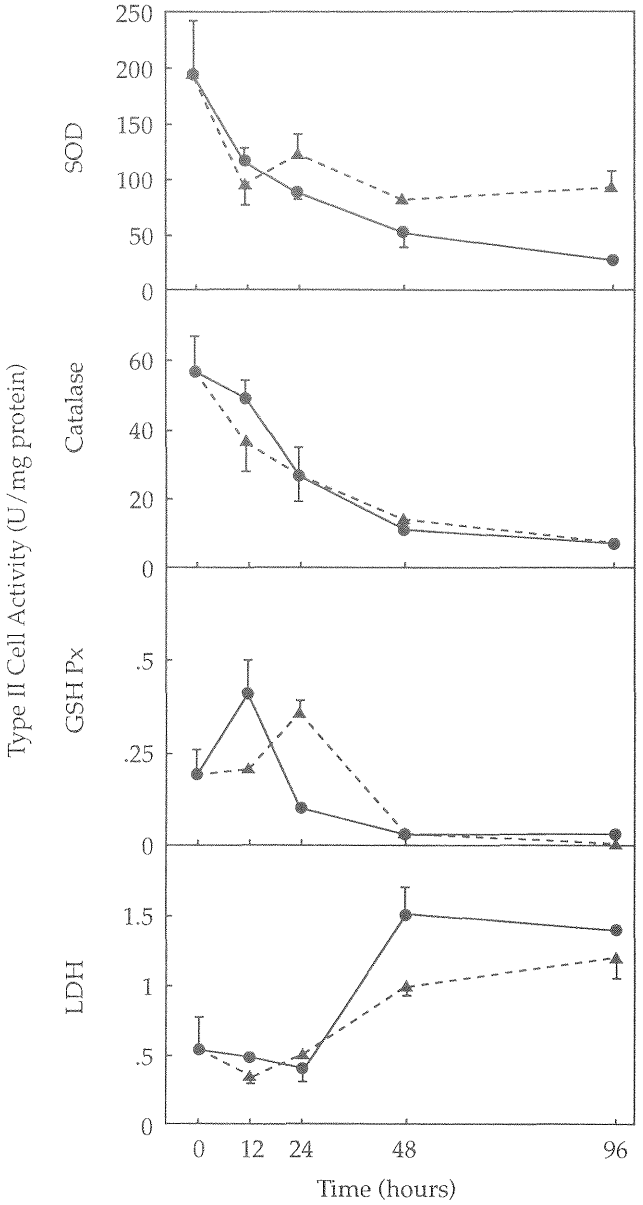

Figure 2. Changes in cultured rabbit type II cell–associated antioxidant enzyme activities during normoxic (●) and hyperoxic (▲) conditions, as stated in Figure 1. The figure demonstrates changes in cellular SOD, catalase, GSH Px, and LDH activities during exposure. Enzyme activities were normalized for culture-associated protein content. Time points and graphs presented in this figure are as for Figure 1, except that GSH Px activity after 48 hours of normoxia was a single measurement.

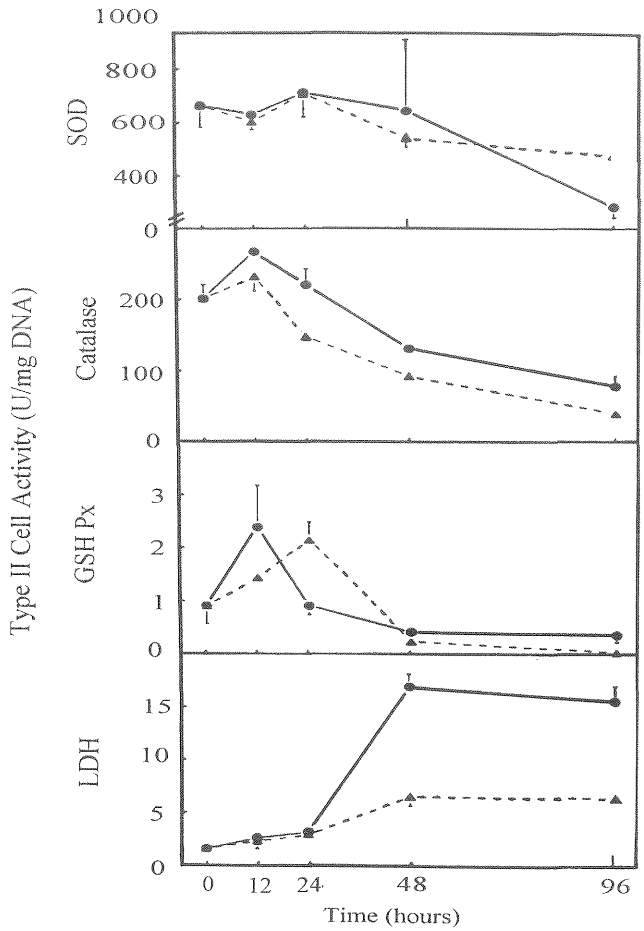

Figure 3. Changes in cultured rabbit type II cell–associated antioxidant enzyme activities during normoxic (●) and hyperoxic (▲) conditions, as stated in Figure 1. The figure demonstrates changes in cellular SOD, catalase, GSH Px, and LDH activities during exposure. Enzyme activities were normalized for cell DNA content. Time points and graphs presented in this figure are as for Figure 1, except that GSH Px activity after 48 hours of normoxia was a single measurement.

Type II cells underwent morphologic changes during exposure to normoxia and hyperoxia as well. Phase contrast microscopy demonstrated that cells subjected to normoxia lost their cuboidal appearance and proceeded to flatten out and increase their cell surface area. Cells also lost their lamellar bodies, as demonstrated by decreased fluorescence when stained with phosphine-3R. Cells subjected to hyperoxia also underwent similar morphologic changes. In addition, cells exposed to hyperoxia demonstrated cytoplasmic swelling within 48 hours of exposure, and within 96 hours of exposure cell detachment was apparent.

Our data show that exposure of type II cultures to hyperoxia for 48 hours was toxic, whereas the exposure with normoxic controls was not. Therefore, another series of experiments was conducted to determine the magnitude of these morphologic changes (Table 2). The results of these experiments support the findings of other experiments as well (Figures 1 through 4). Hyperoxia suppressed cell-associated protein content more than DNA, as demonstrated in Figure 1. As shown in Figures 2 and 3, the depression in LDH activity was greater when normalized for cellular DNA. The data in Table 2 also confirm the data in Figures 2 and 3, indicating that hyperoxia, compared with

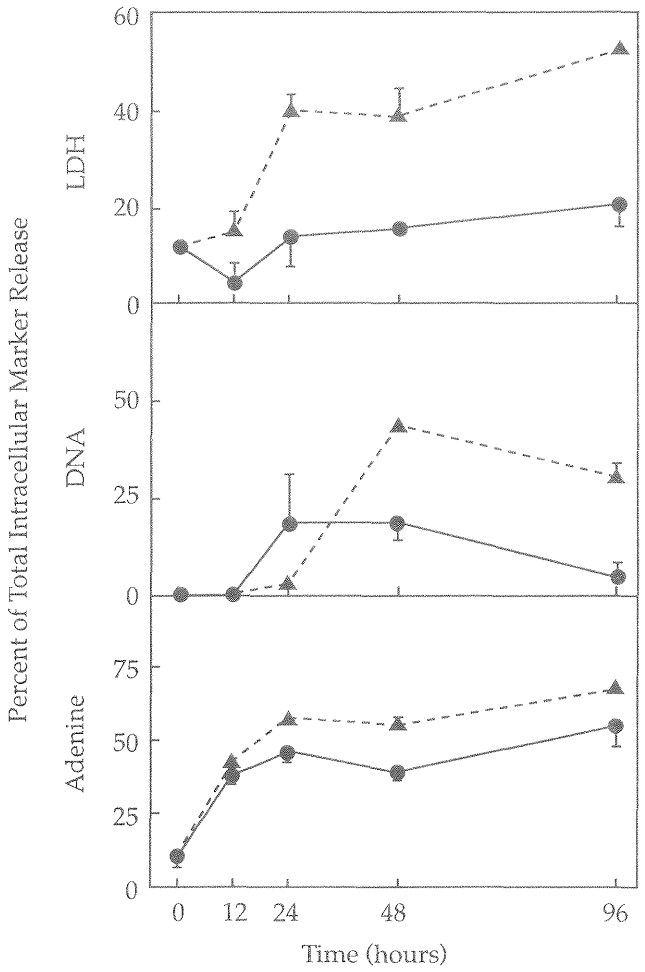

Figure 4. Cell-free LDH activity, DNA, and preincorporated 8-[^{14}C]adenine released from cultured rabbit type II cells under normoxic (●) and hyperoxic (▲) conditions, as stated in Figure 1. Values for each indicator are expressed so that the percentage of cell-free LDH = [(extracellular)/(extracellular + cell-associated)] × 100%.

normoxia, did not significantly alter the rate of loss of cellular catalase or GSH Px activities. Cellular SOD activity in hyperoxic cultures, when normalized for protein, was elevated above that of the 48-hour normoxic counterparts (Table 2). Superoxide dismutase activity normalized for DNA in the hyperoxic cultures after 48 hours of exposure was not significantly increased compared with activity for normoxic cells, although the mean SOD activity of hyperoxic cells was still elevated. Thus, normalizing the SOD activity for cellular DNA rather than for protein resulted in a loss of statistical significance for the increased SOD activity observed in hyperoxic cells. Figure 4 and Table 2 demonstrate that extracellular DNA and 8-[^{14}C]adenine metabolites, both markers of cellular injury, were increased in hyperoxic cultures. The expected release of LDH from cells exposed to hyperoxia was difficult to demonstrate. Increased extracellular LDH release cultures exposed to hyperoxia for 48 hours was marginal compared with increases for normoxic controls (analysis of variance $F \leq 0.10$). In contrast, cell-associated LDH activity in cultures exposed to 95% O_2 for 48 hours was significantly more depressed than that for normoxic controls (Table 2). The depression in cellular LDH activity in the hyperoxic-exposed cultures was greater when normalized for cellular DNA rather than protein (Table 2).

TYPE II CELL CYTOTOXICITY MARKERS AND THEIR RESPONSE TO OXIDANT STRESS

During the course of these experiments, we learned more about the properties of standard markers of cell injury applied to type II cell and oxidant-oriented investigations. The release of intracellular markers such as ^{51}Cr or 8[^{14}C]-adenine prelabels or cytosolic LDH are typical approaches for assessing in vitro cell injury. Comparing ^{51}Cr prelabel and LDH release from type II cells showed differences in the basal rate of release of these cytotoxicity markers into the culture medium (Table 3). In control cells incubated for up to three hours in Hank's balanced salt solution, the fraction of total monolayer LDH release ranged from 5% to 10%, and ^{51}Cr release ranged from 30% to 40% of the total monolayer label. The release of ^{51}Cr increased significantly ($p < 0.05$) after a 3-hour incubation, whereas LDH release did not occur during this period.

Because the type II cell is a secretory cell, the high rate of ^{51}Cr release observed may have been related to associated processes. To test this hypothesis, cells were treated with 20 μM isoproterenol, a β-adrenergic agonist. Previous in vitro studies of type II cells have demonstrated stimulation of surfactant release by these isoproterenol concentrations (Dobbs and Mason 1979; Brown and Longmore 1981). Cells treated with 20 μM isoproterenol exhibited a significantly greater release of ^{51}Cr after two hours of incubation than control cells, with no effect on LDH release (Table 3). Because the release of 10% LDH was consistent with viability determinations of approximately 90%, based on the assessment of cellular trypan blue exclusion, LDH appeared to be a better index of cytotoxicity in the type II cell.

Thus, for a number of our observations of oxidant injury to type II cells and the protective effects of liposome-delivered antioxidants, LDH release served as a marker for cell injury (Figures 9, 10, and 11). Then, we noticed that the expected cell LDH content in hyperoxic-exposed cultures, after analysis of both cell monolayers and culture medium, could not be fully accounted for. It was hypothesized that the enhanced rates of cell $O_2^{\cdot-}$ and H_2O_2 generation inactivated this enzymatic marker of cytolysis. Incubation of purified LDH with xanthine plus XO or H_2O_2 resulted in

Table 2. Phenotypic Changes Induced by Culture of Rabbit Type II Cells for 48 Hours in Air or 95% Oxygen[a]

Response	Treatment		Duncan Test Performed	n
	Normoxia	Hyperoxia		
Cellular ($\mu g/cm^2$)				
DNA	1.4 ± 0.2	1.2 ± 0.2	Yes	7
Protein	15 ± 3	9 ± 2	Yes	7
Protein:DNA	10 ± 2	8 ± 1	No	7
Percentage of total cell medium				
LDH	22 ± 4	43 ± 10	Yes	7
Adenine	53 ± 8	67 ± 8	Yes	7
DNA	17 ± 3	41 ± 2	Yes	7
Enzymatic activity (U/mg protein)				
SOD	57 ± 18	137 ± 20	Yes	7
Catalase	23 ± 3	23 ± 6	No	7
GSH Px	0.11 ± 0.04	0.10 ± 0.03	No	**[b]
LDH	0.75 ± 0.21	0.55 ± 0.15	Yes	7
Enzymatic activity (U/mg DNA)				
SOD	670 ± 232	1,040 ± 167	No	7
Catalase	220 ± 41	204 ± 66	No	7
GSH Px	1.29 ± 0.32	0.97 ± 0.33	No	**
LDH	8.4 ± 2.6	4.5 ± 0.37	Yes	7

[a] The data represent quantitative analyses of type II cell monolayer protein, DNA content, and cellular antioxidant enzyme activities after 48 hours of normoxia or hyperoxia. Cells were allowed to adhere for 39 hours to 9.6-cm^2 dishes, as described in Table 1, before initiation of the experiment. The data represent the sum of three experiments: two conducted in duplicate and the third conducted in triplicate. Data were analyzed via a two-way ANOVA, utilizing a block design to account for experiment-to-experiment differences within a treatment. If in the ANOVA the treatment variable accounted for significant variation in a given dependent response, a Duncan post hoc, groupwise comparison was performed to determine significant differences ($p < 0.05$) between treatments. Each value represents the mean ± SEM.

[b] ** = for normoxia $n = 3$, and for hyperoxia $n = 5$.

inactivation of LDH (Figure 5). A total of 248 μM H_2O_2, final concentration, was added to LDH in solution in equal amounts at zero, two, and 4 hours. In a separate incubation of LDH, XO activity was maintained under substrate-saturating conditions at 3.1 mU/mL. The addition of xanthine and XO resulted in a threefold greater loss of enzymatic activity than a similar incubation with H_2O_2 (Figure 5). The difference in the rate of loss of LDH activity between the two groups may be due to the combined presence of $O_2^{\cdot-}$ and H_2O_2 in the XO exposure. Alternatively, the xanthine and XO system, assuming 100% dismutation of $O_2^{\cdot-}$ to H_2O_2, produced a maximum of 744 μM H_2O_2 during the exper-

Table 3. Release of ^{51}Cr Prelabel and Lactate Dehydrogenase by Cultured Rabbit Type II Cells after Treatment with Isoproterenol

% Total Release	Isoproterenol (μM)	Incubation Time[a] (hours)		
		1	2	3
^{51}Cr	0	31.3 ± 1.2	31.3 ± 0.7	39.4 ± 1.2[b]
	20	32.9 ± 1.4	39.4 ± 1.9[c]	42.2 ± 1.8
LDH	0	9.8 ± 1.9	9.2 ± 2.8	6.0 ± 0.9
	20	9.3 ± 1.4	7.9 ± 1.9	7.9 ± 1.9

[a] Values are means ± SEM for 5 wells containing cells that were incubated with and without 20 μM isoproterenol.

[b] Values are significantly different from cells incubated in the absence of isoproterenol for 1 hour ($p < 0.05$).

[c] Values are significantly different from cells incubated in the absence of isoproterenol for 2 hours ($p < 0.05$). Data were analyzed by ANOVA followed by a Duncan post hoc groupwise comparison procedure.

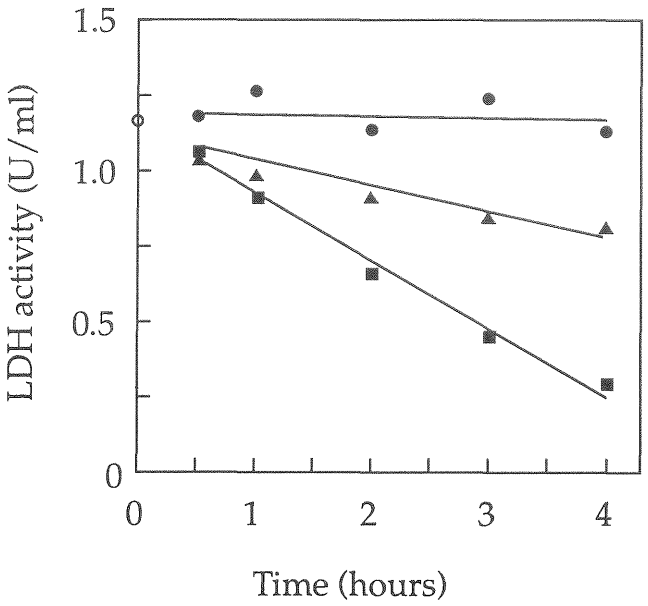

Figure 5. Oxidant inactivation of LDH. Inactivation of purified LDH was initiated by hydrogen peroxide (▲) at a final concentration of 82.5 μM added at 0, 2, and 4 hours, or by 500 μM xanthine plus xanthine oxidase (■), maintained at 3.1:1.3 mU/ml, and was compared to control LDH (●) activity. The open circle (○) on the y axis is the LDH activity at $t = 0$. All groups were incubated at 25°C, and each point represents the average of at least two separate measurements. Slopes of the regression lines from the experimental groups were significantly different from controls ($p < 0.01$). Regressions for ●, ▲, and ■ were $(-0.006)X + 1.19$, $(-0.084)X + 1.12$, and $(-0.225)X + 1.15$, respectively.

iment, which was 2.7-fold greater than the total amount H_2O_2 added directly. The loss of LDH activity was not attributed to dilution of LDH by addition of xanthine and XO or H_2O_2. At the end of four hours, the loss of LDH activity by dilution due to added H_2O_2 or xanthine and XO could only account for 0.28% or 3.24% of the activity loss respectively seen in these groups. From this, we assumed type II cell LDH was oxidatively inactivated in oxidant-stressed cells. ^{14}C-Adenine release, which is not protein- or lipid-associated upon cell uptake and not subject to artifact during surfactant secretory processes, was then selected as our marker of cell injury.

TYPE II CELL UPTAKE OF LIPOSOMAL ANTIOXIDANT ENZYMES

Cationic phospholipids or amphiphiles have been reported to be the most effective liposomal components for in vivo pulmonary delivery of antioxidant enzymes (Panus et al. 1988). In vitro, cationic liposomes also associated more efficiently with pulmonary-derived type II cells (Table 4) than similar anionic or neutral liposomes. Thus, both in vitro and in vivo results suggest that maximal pulmonary delivery and cellular incorporation of macromolecules occurs using cationic liposomes. Type II cell-associated catalase and SOD activities are increased after in vitro incuba-

Table 4. Role of Liposomal Charge in Liposomal Interactions with Rabbit Type II Cells and the Culture Dish Surface[a]

Liposomal Charge	*n*	% Association of Added Liposomal Phospholipid/cm^2
Cationic		
Adherent type II cells	8	3.0 ± 0.43
Culture dishes pretreated with fibronectin	4	0.94 ± 0.07[b]
Untreated culture dishes	4	1.20 ± 0.19[b]
Anionic		
Adherent type II cells	8	1.70 ± 0.50[c]
Culture dishes pretreated with fibronectin	4	0.53 ± 0.06[b]
Untreated culture dishes	4	1.30 ± 0.05
Neutral		
Adherent type II cells	7	0.53 ± 0.30[c]
Culture dishes pretreated with fibronectin	4	0.10 ± 0.02[b]
Untreated culture dishes	4	0.35 ± 0.05

[a] Cells were plated on Costar 9.6-cm^2 dishes for 36 hours (37°C) at a concentration of 4.34×10^5 cells/cm^2. Dishes were pretreated with fibronectin to enhance type II cell adherence and to compare the noncellular interaction of liposomes with fibronectin-pretreated culture dishes and untreated culture dishes. Each value represents the mean ± SEM.

[b] Values are significantly different from adherent type II cells within each liposomal charge species.

[c] Values are significantly different from cationic liposomal uptake by adherent type II cells; $p < 0.05$ by ANOVA followed by Duncan post hoc groupwise comparison procedure.

Table 5. Liposomal Augmentation of Antioxidant Enzymes in Rat Type II Cells[a]

Enzyme	*n*	Specific activity (U/mg DNA)	% Cell Association of Added Liposomal Phospholipid/cm^2
SOD			
Untreated	4	14 ± 8	–
Control liposomes	4	7 ± 4	12 ± 1
SOD liposomes	4	294 ± 106[b]	18 ± 3
Catalase			
Untreated	2	143 ± 10	–
Control liposomes	3	104 ± 25	20 ± 2
Catalase liposomes	3	1,710 ± 459[b]	21 ± 2

[a] The untreated group consisted of control cells not receiving liposomes, whereas the other groups received liposomes, as described in Table 4. Catalase and SOD liposomes were prepared by the method described in Table 4, except that the appropriate enzyme (≈10 mg/mL) was added prior to liposomal formation. Cells were incubated with liposomes (4 hours, 37°C) at initial concentrations of 55 and 52 nmol liposomal phospholipid/cm^2 for controls and enzyme-containing liposomes in the SOD experiment, and 32 and 29 nmol liposomal phospholipid/cm^2 for controls and enzyme-containing liposomes in the catalase experiment. At the termination of the experiment, plates were washed twice in Hank's balanced salt solution, sonicated, and DNA analysis and enzyme analysis were performed. Each value represents the mean ± SEM.

[b] $p < 0.05$ by ANOVA followed by a Duncan post hoc groupwise comparison procedure.

tion with cationic liposomes containing the appropriate enzyme (Table 5). The increased antioxidant enzyme activity of cell monolayers, after incubation of type II cells with liposomes, is not exclusively due to cellular binding and uptake of liposomes, since some liposome association with the cell growth surface also occurs (Table 4). Thus, binding of liposomes to noncellular sources must be considered as well when examining cellular metabolic properties after liposome delivery of macromolecules to subconfluent cells. For example, toxic effects of extracellular oxidants on cultured cells may be attenuated by the oxidant scavenging of liposomally entrapped antioxidant enzymes attached to cell culture dish surfaces, as well as by scavenging from similar intracellular enzymes. Appropriate controls, such as monitoring extracellular levels of antioxidant enzymes, should be conducted when examining liposome-mediated protection of cells from extracellular oxidants.

During our studies, we noticed that catalase was capable of absorbing to the exofacial surface of cationic liposomes during preparation and washing because of favorable electrostatic interactions. Bovine CuZn SOD did not associate with liposomal surfaces and was only accessible for assay after detergent disruption of liposomes. Because of the observation of catalase-membrane association, cells that bound liposomes with catalase adsorbed to the exofacial surface may have exhibited augmented catalase activity in both plasma membrane–associated and intracellular compartments. To better characterize the uptake of liposomal catalase by type II cells, trypsin-resistant cellular catalase activity was measured and compared with total cell-associated catalase activity. A study conducted by Chander and associates (1983) suggested that trypsin-releasable and trypsin-resistant liposomes binding to type II cells represent surface-associated and intracellular pools, respectively. Catalase activities of cells that were harvested by either scraping or trypsinization in the present study are reported for control cells and cells incubated for two hours with catalase liposomes at a concentration of 400 nmol phospholipid/9 cm^2 (Table 6). Incubation of cells with catalase liposomes increased total cellular catalase-specific activity to 568% of that of controls, and trypsin-resistant catalase activity to 213% that of controls. Trypsin-resistant catalase activity represented more than 80% of total catalase activity in control cells and 30% of the total in catalase liposome-treated cells. These data suggest that, in addition to significant internalization of liposomal catalase, much of the liposomal catalase remained associated with the cell surface after a two-hour incubation period.

Table 6. Total and Trypsin-Resistant Rat Type II Cell Catalase Activities[a]

Treatment	Catalase (U/mg Cell Protein)
Control	
Scraped	89 ± 3
Trypsinized	72 ± 2
Catalase liposomes	
Scraped	505 ± 38[b]
Trypsinized	153 ± 17[b]

[a] Liposome-treated cells were incubated with 400 nmol phospholipid/9 cm^2 for 2 hours. Liposomes contained 2,008 U catalase/μmol phospholipids or 9,833 U catalase/mg liposome-entrapped protein. Cells were harvested by scraping or trypsinization for determination of total and trypsin-resistant activity, respectively. Each value represents the mean ± SEM for 5 to 6 dishes of cells.

[b] $p < 0.05$, compared with similarly harvested control cells.

Intracellular delivery of liposomal antioxidant enzymes to alveolar type II cells plated at a low seeding density was demonstrated by fluorescence microscopy, using liposome-entrapped fluorescein isothiocyanate (FITC)–labeled CuZn SOD (Figures 6 and 7). Type II cells prelabeled with liposome-entrapped FITC-SOD showed both intracellular punctuate and diffuse fluorescence, suggesting binding or uptake or both of intact liposomes and dispersion of liposomal contents throughout the cytoplasm. No detectable free association of FITC-SOD with cells occurred. Cells treated with empty liposomes or nonfluorescent SOD showed no fluorescence (data not shown). In our experiments, cells treated with up to 1 μmol liposomal phospholipid/cm^2 exhibited no toxicity due to the vehicle and incorporated up to 10% of the added liposomes during a two-hour incubation.

TYPE II CELL UPTAKE OF LIPOSOMAL α-TOCOPHEROL

The amphiphilic oxidant scavenger α-tocopherol was incorporated into liposomal membranes efficiently, after it was added to the standard lipid mixture used for the preparation of liposomes. Direct analysis of liposomal membrane α-tocopherol content showed that 66% to 90% of the added α-tocopherol was present in final washed liposomal suspensions, representing up to 28 mol% (a percent ratio of components on a mol to mol basis) of liposomal phospholipids (Table 7). Possible alterations in biophysical characteristics of liposomal membranes by α-tocopherol did not significantly affect type II cell uptake of liposomes (Table 8). It was observed that maximum cell incorporation of liposomal α-tocopherol occurred within four hours of exposure (Figure 8), resulting in up to a 20-fold enhancement

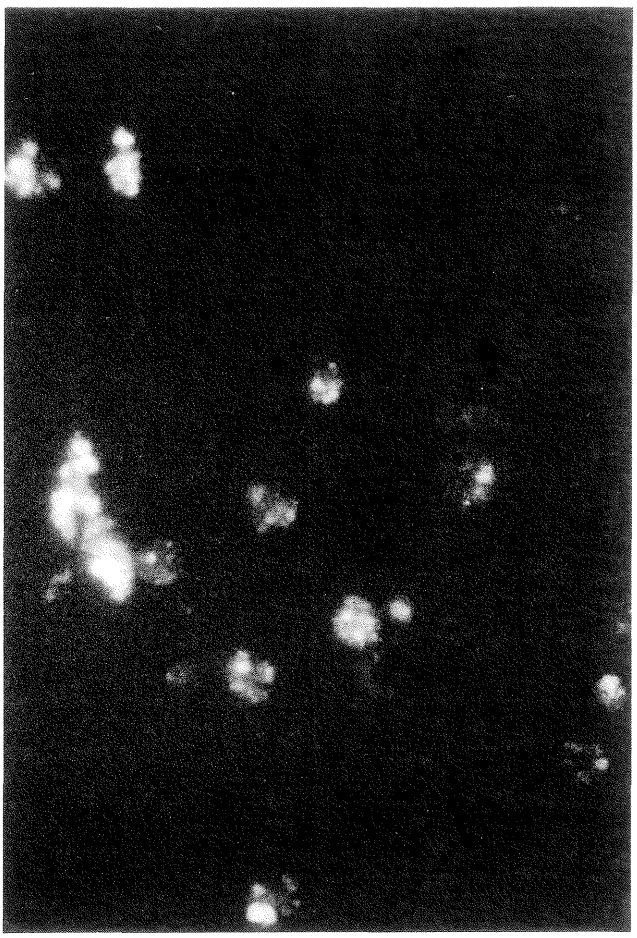

Figure 6. A representative fluorescent micrograph of rabbit type II cells 23 hours after delivery of liposomal FITC-SOD. Cells were isolated and plated at low density. Magnification is × 1,475.

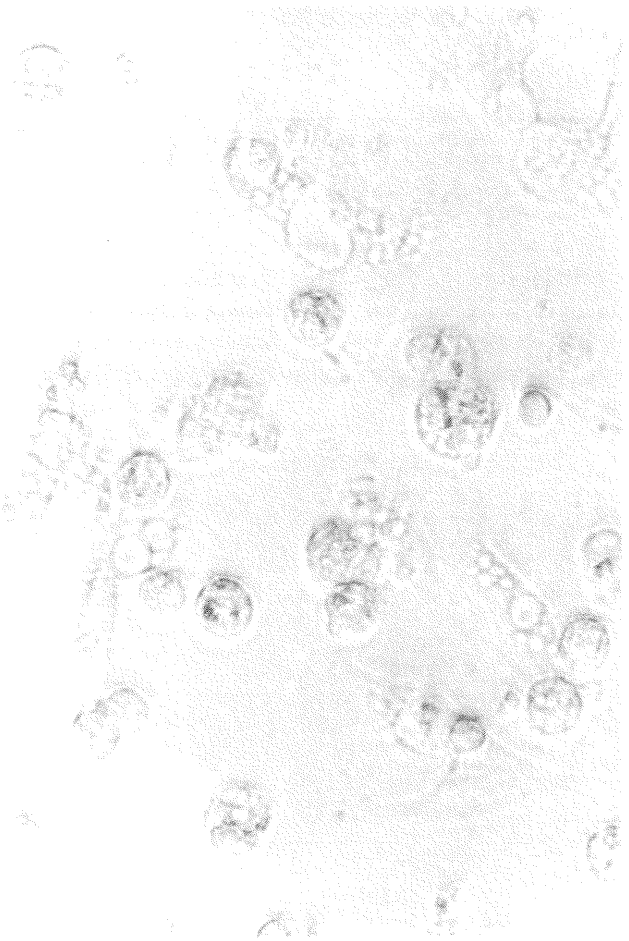

Figure 7. The corresponding phase contrast micrograph of Figure 6. Magnification is × 1,475.

Table 7. Liposomal Ratios of α-Tocopherol Supplementation and Incorporation

Preparation	DPPC	Cholesterol	Stearylamine	Added α-Tocopherol	Measured mol Ratio of Liposomal α-Tocopherol
1	7	1	2	0	0
2	7	1	2	0.5	0.4
3	7	1	2	1	0.9
4	7	1	2	2	1.3
5	7	1	2	3	2.0

[a] Liposomes were prepared by reverse-phase evaporation as described in the Methods section, with increasing mol ratios of α-tocopherol. Direct fluorometric analysis of liposomal α-tocopherol content shows the actual mol ratio of α-tocopherol incorporated into liposomal membranes.

Table 8. The Influence of Liposomal α-Tocopherol Content on Cultured Rabbit Type II Cell α-Tocopherol Uptake[a]

Preparation	DPPC:CHOL:SA:α-Toc[b] (mol:mol)	μg α-Tocopherol per mg Cell Protein	α-Tocopherol Uptake (%)	% Liposomal Uptake (%)
1	-	38	-	-
2	7:1:2:0	29	-	27
3	7:1:2:0.4	66	29	22
4	7:1:2:0.9	107	23	20
5	7:1:2:1.3	199	20	18
6	7:1:2:2.0	201	12	14

[a] Increasing mol ratios of α-tocopherol were incorporated into liposomal membranes. Then, type II cell monolayers were similarly treated with 100 nmol liposomal phospholipid/cm^2 cells for 4 hours. Cell monolayers were then assayed for extent of α-tocopherol and liposomal uptake.

[b] Dipalmitoylphosphatidylcholine:cholesterol:stearylamine:α-tocopherol.

of cellular α-tocopherol content. In a 4-hour incubation of type II cells with a medium containing α-tocopherol–supplemented serum, there was no detectable increase in cell α-tocopherol content. In contrast, addition of liposomes containing α-tocopherol to the culture medium resulted in a significantly greater enrichment in cell α-tocopherol (Table 9).

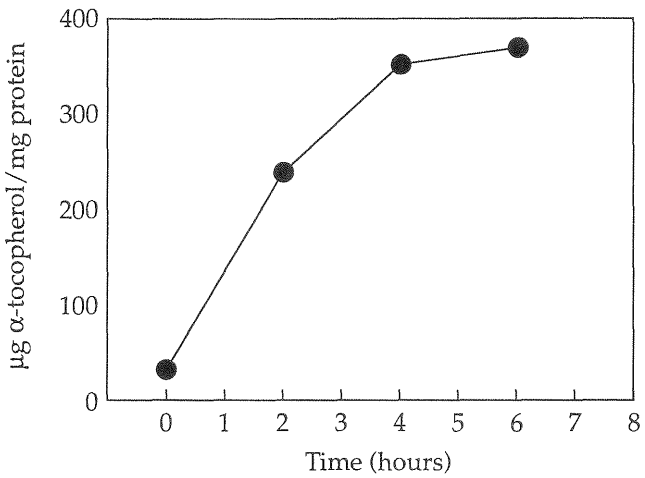

Figure 8. Time course of liposomal α-tocopherol uptake by cultured rabbit type II cells. Cells were treated with liposomal α-tocopherol, in the method described for Table 9.

PROTECTION OF OXIDANT-STRESSED TYPE II CELLS WITH LIPOSOME-DELIVERED ANTIOXIDANTS

After a six-hour exposure to oxidants generated by XO or glucose oxidase added to culture medium, LDH release was significantly less in catalase liposome–pretreated and washed cells than in controls. Partial protection against oxidant injury induced by XO (Figure 9) and complete protection against glucose oxidase injury (Figure 10) were observed after pretreatment with catalase liposomes, as indicated by attenuated cell LDH release. Catalase liposome–pretreated cells also were protected from detachment from monolayers induced by oxidant exposure. Oxidant-mediated injury was not decreased by pretreating cells with empty liposomes. In the absence of glucose oxidase, up to 400 nmol phospholipid/9 cm^2 of empty or catalase liposomes was not associated with increased cytotoxicity (Figure 10). The addition of native catalase to the culture medium prevented the glucose oxidase–mediated cytotoxicity and the presumably

Table 9. Serum Versus Liposomes as a Vector for α-Tocopherol Delivery to Cultured Rabbit Type II Cells

Condition[a]	μg α-Tocopherol per mL Medium	μg α-Tocopherol per mg Protein
Medium + α-tocopherol	9.1	Not detectable
Medium + α-tocopherol liposomes	22.1	440

[a] Fetal calf serum, which had undetectable α-tocopherol, was supplemented with an ethanolic solution of α-tocopherol. Cells were incubated for 4 hours with α-tocopherol–supplemented medium containing 10% fetal calf serum or with unsupplemented medium plus liposomes containing α-tocopherol (dipalmitoylphosphatidycholine:cholesterol:stearylamine:α-tocopherol; 7:1:2:2, mol:mol). Then, cell monolayer lipids were assayed fluorometrically for α-tocopherol content. Endogenous cell α-tocopherol content was 28 μg/mg cell protein.

H_2O_2-dependent component of XO-mediated cytotoxicity. The effectiveness of native catalase was probably due to its immediate scavenging of enzyme-derived H_2O_2 in the culture medium before it reacted with type II cells to induce LDH release.

Pretreatment of cells with 50, 100, and 200 nmol liposomal phospholipid/9 cm^2 resulted in augmentation of cellular catalase activities to levels 182%, 252%, and 569% greater than that of controls, respectively (Figure 11). After a six-hour exposure of cells to a mixture of glucose and glucose oxidase, type II cell LDH release into culture medium was studied in these catalase liposome–treated cells. Control cells released 70% of total intracellular LDH, whereas cells pretreated with catalase liposomes with 50, 100, and 200 nmol phospholipid/9 cm^2 released 58%, 37%, and 13% of the total LDH, respectively. A plot of type II cell catalase-specific activity against the percentage of LDH release for control cells and catalase liposome–treated cells demonstrates a correlation coefficient of 0.95 for this relationship.

Confirmation that catalase liposome–treated cells were scavenging H_2O_2, thereby preventing cell injury, was derived from measurements of media H_2O_2 concentrations

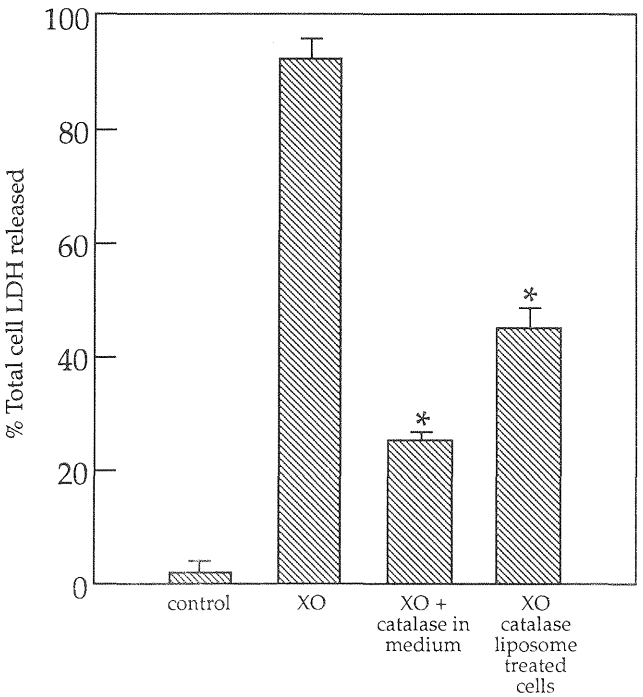

Figure 9. Catalase liposome–mediated protection of rat type II cells against XO injury, as measured by LDH release. Cells were pretreated for 2 hours with 0 or 50 nmol phospholipid/cm^2 catalase liposomes, containing 3,622 U catalase/mol phospholipid or 9,183 U catalase/mg liposome-entrapped protein, and washed. A six-hour incubation of cells in HBSS containing 0.1 mM xanthine and 50 mU/ml XO followed. Extracellular catalase (670 U catalase/mL) was present in one treatment group during a six-hour incubation with XO. Results are means ± SEM for 5 dishes containing cells incubated under each condition. An * indicates values significantly different from control values for cells incubated with XO ($p < 0.05$) by ANOVA followed by the Duncan multiple comparison procedure.

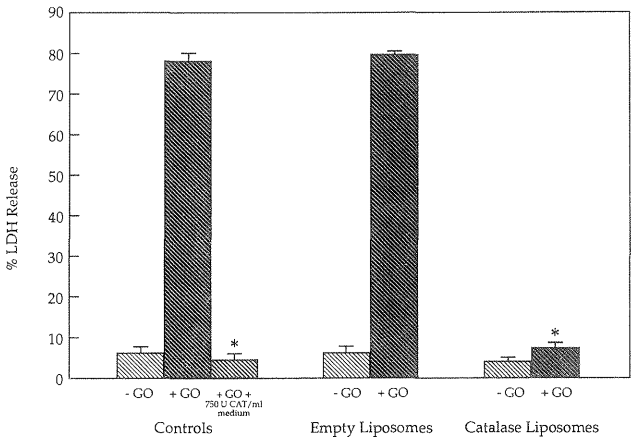

Figure 10. Catalase liposome–mediated protection of rat type II cells from glucose oxidase injury, as measured by LDH release. Cells were pretreated for 2 hours with 0 or 50 nmol liposome phospholipid/cm^2 of empty or catalase liposomes, and washed. Catalase liposomes contained 2,598 U catalase/μmol phospholipid or 7,094 U catalase/mg liposome-entrapped protein. A 6-hour incubation of cells in HBSS containing 11.2 mM glucose, and treatment with 0 or 200 mU/ml glucose oxidase was performed. Extracellular catalase (CAT) (750 U/mL) was present in one treatment group during 6-hour incubation with glucose oxidase. Results are means ± SEM for 4 to 5 dishes of cells incubated under each condition. An * indicates values significantly different from control values for cells incubated with glucose oxidase ($p < 0.05$) by ANOVA followed by Duncan multiple comparison procedure.

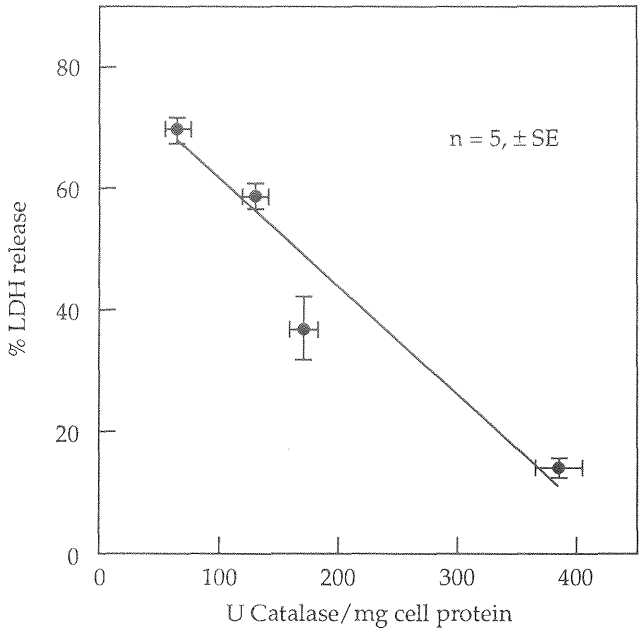

Figure 11. Dose response of catalase liposome-mediated protection of rat type II cells from glucose oxidase injury. Cellular catalase specific activity is plotted against the percentage of LDH release. Cells were pretreated with 0, 5, 10, and 20 nmol phospholipid/cm^2 for 2 hours and washed. Catalase liposomes contained 2,598 U catalase/μmol phospholipid or 15,422 U catalase/mg liposome-entrapped protein. A 6-hour incubation of cells in HBSS containing 11.2 mM glucose, and treatment with 0 or 200 mU/mL glucose oxidase followed. Results are means ± SEM for 2 parallel sets of 4 dishes containing cells incubated under each condition.

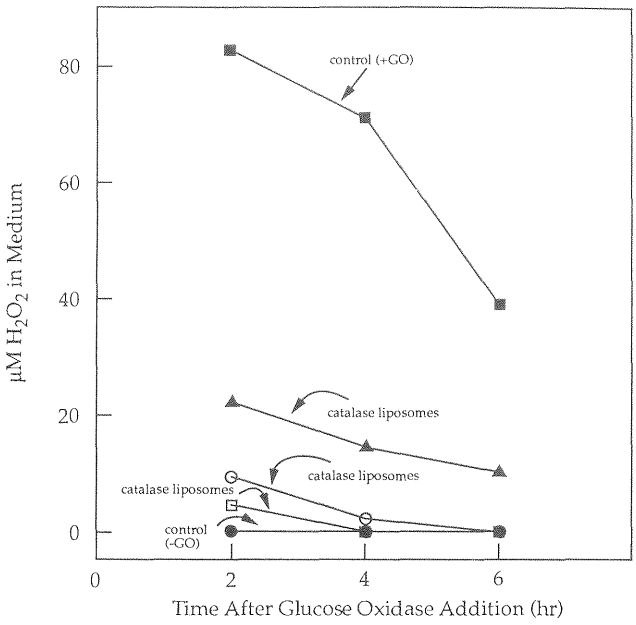

Figure 12. H_2O_2 scavenging by glucose oxidase-challenged controls and catalase liposome-treated rat type II cells. Controls are designated by filled squares (■) and circles (●) in the presence and absence of glucose oxidase, respectively. Cells challenged with glucose oxidase were pretreated with 5 (▲), 10 (○), and 20 (□) nmol phospholipid/cm^2 catalase liposomes in the method described in legend of Figure 11. Results represent concentrations of H_2O_2 measured in medium of type II cells incubated in HBSS containing 11.2 mM glucose and 0 or 200 mU/ml glucose oxidase.

during exposure of cells to the mixture of glucose and glucose oxidase (Figure 12).

PULMONARY DELIVERY OF LIPOSOME-ENTRAPPED SUPEROXIDE DISMUTASE AND CATALASE

A single intratracheal instillation of liposomes containing both CuZn SOD and catalase increased the specific activities of these enzymes in both lung homogenates (Figures 13 and 14) and isolated type II cells (Figure 15) from treated rabbit lungs. Lung homogenate CuZn SOD and catalase activities increased two hours after liposome instillation (Figures 13 and 14), with peak activity reached by four hours. Then, CuZn SOD activity decreased toward control levels, whereas catalase activity remained elevated. Twenty-four hours after liposome instillation, lung CuZn SOD–specific activity was not different from that of controls, whereas catalase activity was still 170% elevated. As expected, there were no significant changes in lung Mn SOD activity. There were significant differences in tissue enzyme augmentation of SOD and catalase activities in each lung lobe, with respect to time, for both CuZn SOD ($p<0.05$) (Figure

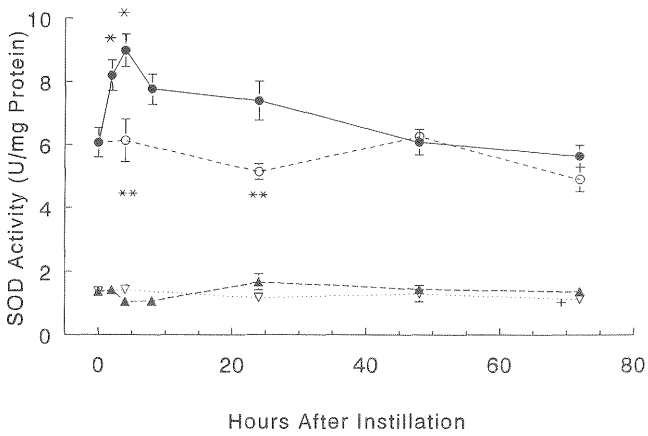

Figure 13. Activity of SOD in rabbit lung homogenate after a single intratracheal instillation of liposomes containing CuZn SOD and catalase. Rabbits were exposed to either room air (●) or 100% O_2 (○) immediately after liposome instillation. An * indicates significantly greater than time = 0; a ** indicates significantly less than the corresponding room air value ($p<0.005$). CuZn SOD activity peaked 4 hours after treatment with liposomes and returned to control levels after 24 hours. Exposure to 100% O_2 significantly decreased augmentation of CuZn SOD activity in the lungs both 4 and 24 hours after liposome treatment. There were no significant changes in Mn SOD activity after liposome instillation and exposure to either air (▲) or 100% O_2 (▽). All values are means ± SEM; $n \geq 15$.

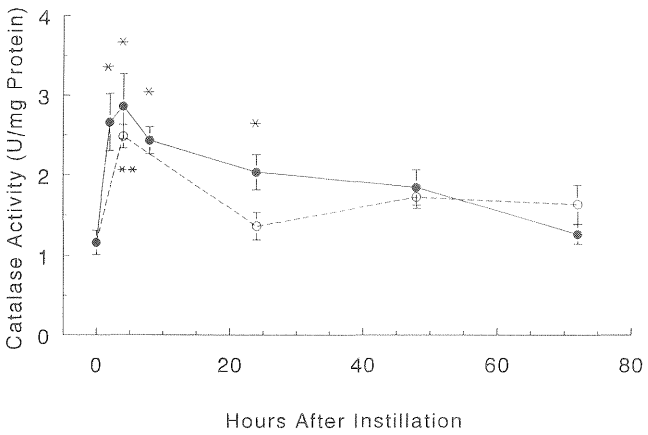

Figure 14. Activity of catalase in rabbit lung homogenate after a single intratracheal instillation of liposomes containing CuZn SOD and catalase. Rabbits were exposed to either room air (●) or 100% O_2 (○) immediately after liposome instillation. Catalase activity was significantly less than that for controls for 24 hours after instillation. Exposure to 100% oxygen had no effect on catalase augmentation by liposome instillation into the lungs. All values are means ± SEM; $n \geq 15$. An * indicates values significantly greater than time = 0; a ** indicates values significantly less than corresponding room air value ($p < 0.005$).

16) and catalase ($p<0.05$) (Figure 17), with similar changes in CuZn SOD and catalase activity with respect to lung lobe ($p>0.12$ and $p>0.34$, respectively). Figure 18 shows an epifluorescence image of a rabbit lung parenchyma four hours after instillation of liposomes containing FITC-SOD. Alveolar structures demonstrated widespread distribution of intracellular fluorescence.

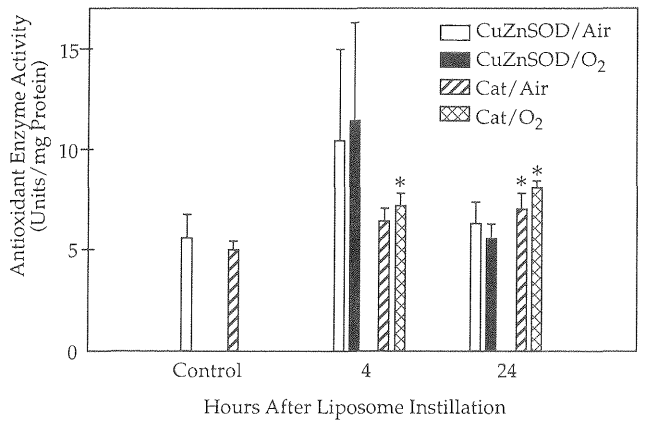

Figure 15. SOD and catalase activity in rabbit type II cells isolated 4 or 24 hours after liposome instillation. Type II cell CuZn SOD activity increased 4 hours after liposome instillation and returned to control levels within 24 hours, as was seen in lung homogenates. However, exposure to 100% O_2 did not decrease CuZn SOD augmentation in type II cells. Catalase activity in type II cells was significantly greater than control levels both 4 and 24 hours after liposome instillation. As in lung homogenates, 100% O_2 did not affect catalase uptake. Results are expressed as means ± SEM, $n \geq 15$. An * indicates values significantly greater than time = 0 ($p<0.005$).

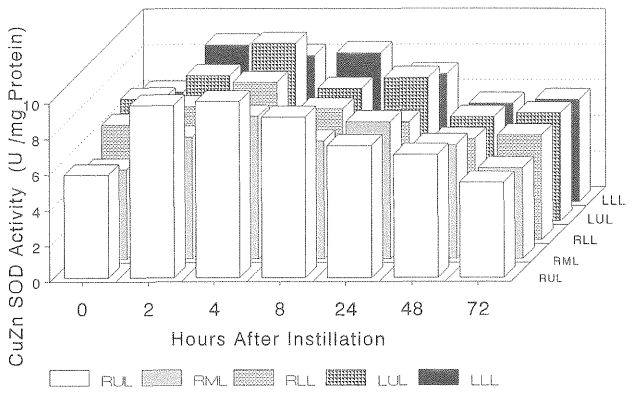

Figure 16. Liposome-mediated augmentation of rabbit lung CuZn SOD activity with respect to time and lung lobar distribution. There are significant differences in augmentation in catalase activity with time (Figure 14). There are no significant differences ($p>0.34$) of catalase augmentation between lobes. Values expressed are means; $n \geq 4$. Data were analyzed by ANOVA, followed by Duncan multiple comparison procedure. RUL: right upper lobe; RML: right middle lobe; RLL: right lower lobe; LUL: left upper lobe; and LLL: left lower lobe.

TYPE II CELL XANTHINE OXIDASE IS A SIGNIFICANT SOURCE OF TISSUE-REACTIVE OXYGEN SPECIES

In the course of our study, it was revealed in the literature that xanthine oxidase is a key source of PROS in vascular endothelium (Rodell et al. 1987). In our efforts to understand oxidant metabolism in type II cells better, we digressed from our original specific aims to investigate the possibility that this enzyme serves as a locus of PROS in type II cells as well, which could readily be amenable to pharmacologic

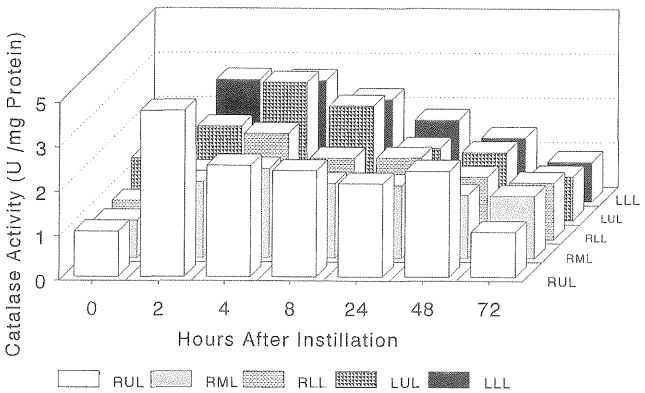

Figure 17. Liposome-mediated augmentation of rabbit lung catalase activity with respect to time and lung lobar distribution. There are significant differences in augmentation of catalase activity with time (Figure 14). There are no significant differences ($p>0.34$) in catalase augmentation between lobes. Values expressed are means; $n \geq 4$. Data were analyzed by ANOVA, followed by Duncan multiple comparison procedure. RUL: right upper lobe; RML: right middle lobe; RLL: right lower lobe; LUL: left upper lobe; and LLL: left lower lobe.

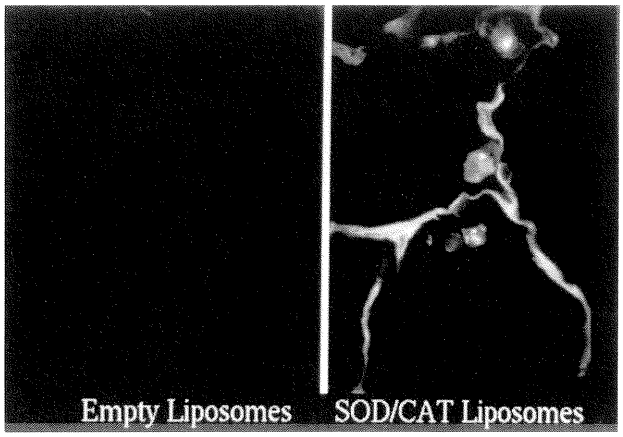

Figure 18. Fluorescence images of frozen rabbit lung sections (1 μm) 4 hours after instillation of empty liposomes or liposomes containing FITC-SOD and catalase (CAT). Enzyme-associated fluorescence intensity was much greater for FITC-SOD than corresponding autofluorescence. Magnification is × 1,000.

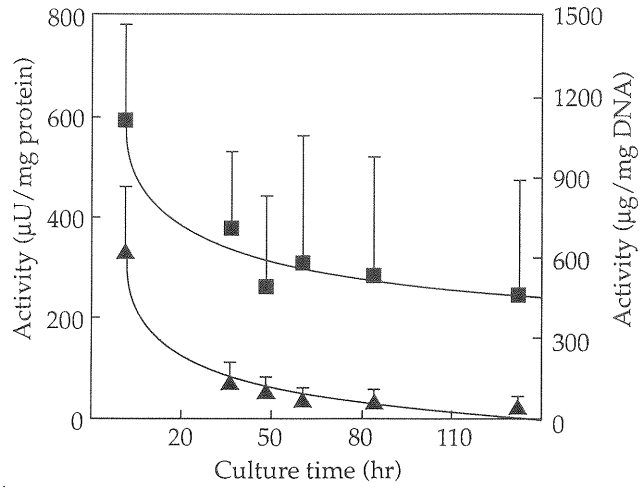

Figure 19. Effects of culture on rat type II cell total XDH and XO activity. Total cellular XDH and XO activity was normalized for protein (▲) and DNA (■). Values normalized for protein and DNA represent the means ± SEM of a minimum of six separate measurements, except at 132 hours, where $n = 5$.

manipulation. Knowing how the dehydrogenase form can be converted to the oxidase by intramolecular thiol oxidation, it is possible that inhaled oxidants can induce formation of the PROS-producing form of the enzyme. Both rat and rabbit lung type II cells had XO activity, with rat type II cells having an eight- to nine-fold greater specific activity than rabbit type II cells (Table 10). It was also observed that rat type II cell XO was unstable during in vitro culture of cells (Figure 19), which was similar to characteristics of the antioxidant enzymes reported in Figures 2 and 3. During the initial 36 hours of culture, loss of XDH/XO activity was 78% and 36%, when normalized for cell protein and DNA, respectively. Loss of cellular XDH/XO activity was not entirely due to an increase in cell protein, because enzymatic activity decreased even when normalized for DNA. The rate of loss of total XDH/XO activity in the cultured type II cells was logarithmic with respect to time when normalized for protein (activity = 324[μU/mg] − 67[μU/mg per hour] • $\log_e$ X) or DNA (activity = 1120[μU/mg] − 136[μU/mg per hour] • $\log_e$ X). In freshly isolated rat type II cells, the percentage of total cellular XDH/XO activity in the oxidase form (percentage of XO) was 31%. During culture, the percentage of type II cell XDH/XO in the oxidase form increased. After 132 hours of culture, the percentage of total enzyme in the XO was 67%. The increase in the percentage of XO during culture demonstrated no clear trend, as determined by an insignificant correlation ($r = 0.734$). Although there was a significant increase in the percentage of XO for type II cells after 132 hours of culture, when compared with changes for freshly isolated cells or cells 36 hours after plating, there was a decrease in total XDH/XO activity (Figure 19). Thus, the net $O_2^{\bullet-}$ and H_2O_2-generating potential of cell XO decreased relative to the amount of time in culture.

Cellular XDH/XO activity was also detected in the cell pellet (200 × g, 5 minutes, 4°C) from bronchoalveolar lavage. The total lavage cell XDH/XO activity was 240 ± 92 mU/mg protein, with 37% ± 7% of the total XDH/XO activity in the oxidase form. In our experiments, type II cell purity determined by phosphine-3R staining was 84%. Thus, total XDH/XO activity from non–type II cell sources (i.e., bronchoalveolar cells) in the final type II cell preparation was a maximum of 38 μU/mg protein, or 12% of the total XDH/XO activity in the final preparation.

Previous investigations have shown that precursors choline and palmitate, when incorporated into the surfactant, are sensitive to oxidant inhibition (Gilder and McSherry 1974; Haagsman et al. 1985; Crim and Simon 1988). We observed that incubation of rabbit type II cells with the XO substrate xanthine significantly decreased choline and palmitate incorporation into phosphatidylcholine by 8% ($p \leq 0.05$, using the one-tailed t test) and 17% ($p \leq 0.05$) of

Table 10. Detection of Xanthine Dehydrogenase and Xanthine Oxidase in Freshly Isolated Type II Cells[a]

Species	n	XDH + XO Activity (μU/mg protein)	XO XO + XDH (%)
Rabbit	5	33 ± 24	25 ± 10
Rat	5	270 ± 199	32 ± 12

[a] Type II cells were isolated and assayed for XDH/XO activity, as described in the Methods section. Data represent means ± SEM.

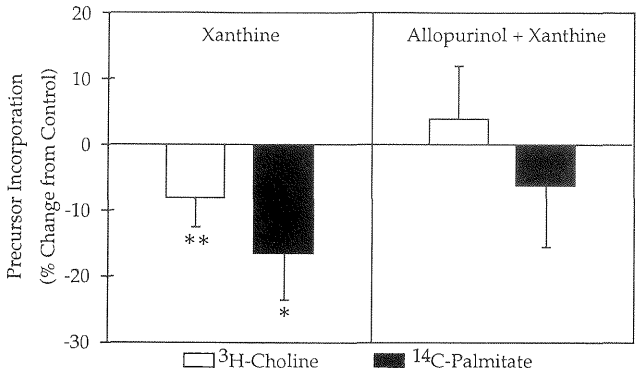

Figure 20. [Methyl-^{3}H]choline and [1-^{14}C]palmitic acid incorporation (% control) into total PC in freshly isolated rabbit type II cells incubated with 500 μM xanthine or 500 μM xanthine plus 10 μM allopurinol. In cells exposed to xanthine alone, both choline (□) and palmitate (■) incorporation were significantly decreased compared with control values. Addition of allopurinol returned precursor incorporation to control values. Data are means ± SEM; $n \geq 6$. An * indicates a value significantly different from corresponding control value, using a one-tailed t test $p \leq 0.05$. A ** indicates a value significantly different from corresponding control value using a two-tailed t test, $p \leq 0.05$.

controls, respectively (Figure 20). Addition of the XO inhibitor allopurinol to the cell suspensions completely inhibited XDH and XO activity and mitigated the decrease in precursor incorporation (Figure 20). This proves a significant modulatory effect of oxidants produced by endogenous type II cell XO on basal rates of surfactant metabolism.

DISCUSSION

A number of important observations were derived from this investigation and can be briefly summarized:

1. We observed a rapid dedifferentiation of endogenous antioxidant defenses of type II cells maintained in vitro. This suggests that these cells do not serve as an appropriate in vitro model for pulmonary type II epithelial cell responses to subacute or chronic oxidant stress.
2. We have demonstrated that liposomes serve as an effective vector for enhancing the antioxidant capacity of pulmonary epithelium in vitro and in vivo.
3. We have observed that XO plays a contributory role in endogenous rates of type II cell production of PROS, which attenuates basal rates of oxidant-sensitive surfactant synthesis in vitro. This shows that PROS affect type II cell differentiated function, reveals a new site of type II cell oxidant production, and represents a possible target for pharmacologic intervention in oxidant lung injury.

The final accomplishments of this investigation differ from the original specific aims. First, we proposed much more than could be realistically studied in a detailed and insightful fashion during the project period. Also, in the course of this work, we discovered that type II cells in vitro would not reliably reflect type II cell differentiated oxidant and antioxidant functions because of drastic in vitro changes in levels of both sources and scavengers of PROS. Thus, it became clear that our experimental observations and manipulations in vitro would be informative as they pertained to and compared with common controls, but in vitro cell culture control and experimental responses would not be predictive of in vivo phenomena. In light of this insight, we emphasized in vitro oxidant response studies less and investigated the role of XO in the type II cell oxidant generation. This endeavor yielded productive and important data that affects our understanding of the biochemical responses of lung cells to environmental oxidant stress. Each of our areas of investigation into type II cell responses to oxidant stress is discussed in detail in the following text.

PHENOTYPIC AND ANTIOXIDANT ENZYME CHANGES IN TYPE II CELLS IN VITRO

The purpose of this series of experiments was to define baseline characteristics of type II cells and their antioxidant defenses in vitro and to reveal responses of these cells to oxidant stress. Thus, type II cells were exposed either to normoxia, a mixture of 95% air and 5% CO_2, or to hyperoxia, a mixture of 95% O_2 and 5% CO_2. In vivo, type II cells maintain constant specific activities of antioxidant defenses in normoxia and respond to hyperoxia with elevated SOD, GSH Px, and catalase activities when normalized for cellular DNA (Freeman et al. 1986). In this study, type II cells exposed to hyperoxia had increased cellular volumes and ratios of protein to DNA. When cell antioxidant enzyme activities were normalized for protein, only CuZn and Mn SOD activities were elevated. In another investigation (Forman and Fisher 1981), adult rats were exposed to 80% O_2 for seven days. Isolated type II cells from these animals demonstrated only increased Mn SOD activity when normalized for DNA and no apparent change in cellular catalase activity. Exposure of rabbits to 64 hours of 100% O_2 followed by seven days in room air resulted in tolerance to subsequent normally lethal hyperoxia exposure (Baker et al. 1989). Type II cells, isolated from these oxygen-tolerant animals, had increased cell volume but no enhanced cellular SOD and catalase activities when normalized for either cellular DNA or protein. Different changes in antioxidant enzyme activities in type II cells isolated from exposed rats and rabbits may be dependent on experimental variations in the hyperoxic exposure or unique biochemical characteristics of the species being examined. Augmentation of type II cell antioxidant enzyme activities may also be influenced by factors released from other cell types. Herein, we deter-

mined cellular responses of type II cells exposed to normoxia and sublethal levels of hyperoxia without the presence of such factors.

Antioxidant enzyme activities (i.e., SOD, catalase, and GSH Px) decreased during 96 hours of exposure to normoxia or hyperoxia. Previous investigations have demonstrated that various forms of partially reduced oxygen inactivate the enzymes mentioned above (Hodgson and Fridovich 1975; Kono and Fridovich 1982; Blum and Fridovich 1985). Superoxide irreversibly inactivates both catalase and GSH Px (Kono and Fridovich 1982; Blum and Fridovich 1985), whereas H_2O_2 inactivates SOD (Hodgson and Fridovich 1975). However, in the present investigation, no definitive mechanism explains the loss of antioxidant enzyme activities in cultured type II cells. In normoxic cultures, the decrease in cellular antioxidant enzymes seems to correspond to the increase in cellular protein (Figures 1 and 2). In fact, if initial catalase, GSH Px, and SOD activities in the normoxic cultures were normalized for changes in cell-associated protein, the predicted values could fall within 57% of values presented in Figure 2. The rate of decrease in cell enzyme activities determined by the predicted values, however, constantly underestimated the actual changes shown in Figure 2. Thus, the predicted values were all at least 33% higher at the end of the experiment. Changes in cellular protein also did not account for the spike in GSH Px activity in the normoxic group after 12 hours of exposure. As seen in Figure 1, no major increase in cell protein occurred in the hyperoxic-exposed group. However, Figure 2 demonstrates that the activities of catalase and GSH Px decreased at rates similar to those of normoxic controls. These results show that the decrease in antioxidant enzyme activities in these cultures was not entirely due to protein dilution. Furthermore, SOD activity in hyperoxic cultures, although greater than normoxic activities, was less than that of predicted values (Figure 2). From the predicted values, the activities of all three enzymes should have been greater than those for controls. However, Figure 2 and Table 2 show that only SOD activity was greater than normoxic activity. If decreases in activities of the antioxidant enzymes in normoxic cultures were influenced by increased culture-associated protein, then activity changes were specific to antioxidant enzymes, because during this same time cellular LDH activity increased in both normoxic and hyperoxic exposures. The reason for the increase in cellular LDH activity in both exposure groups is undetermined at present, but this may be due to culture-induced changes in the type II cells. Also, when SOD, catalase, and GSH Px activities were normalized for cell number, as represented by cellular DNA, the general decrease in activity still occurred. Different mechanisms in hyperoxia and normoxia may control the rates of loss of cellular catalase, GSH Px, and SOD activities. However, culture-induced changes in the phenotypic expression of both groups of cells would seem to be at least one underlying cause. The increase in cellular SOD activity in the hyperoxic group, when compared to normoxic controls, is probably an adaptive response to increased cellular, partially reduced oxygen production during hyperoxic exposure.

This series of experiments demonstrated the cytotoxic effects of hyperoxia on cultured type II cells and suggests that PROS mediated these effects. The intracellular loci producing PROS are probably diverse and at present are only partially characterized. Augmented cellular SOD activity in the hyperoxic-exposed cells was insufficient to protect the cells from the toxic effects of oxygen. The results do not necessarily suggest that selective augmentation of cellular antioxidant enzymes would not be protective, but that in these experiments endogenous rates of cellular PROS production may have overcome cellular antioxidant defenses. The drastic decreases in type II cell antioxidant enzyme activity in vitro of both control and hyperoxia-treated cells suggest:

1. Baseline antioxidant characteristics of these cells in vitro differ from those in vivo.
2. Type II cells respond differently in vitro to hyperoxia than in vivo.
3. Type II cells in vitro may not serve as good models for in vivo pulmonary alveolar epithelial responses to oxidant stress or antioxidant enzyme pharmacology.
4. Dedifferentiated type II cell antioxidant characteristics may resemble type I cell properties.

LACTATE DEHYDROGENASE, ^{51}Cr AND ^{14}C-ADENINE RELEASE AS INDICATORS OF TYPE II CELL OXIDANT INJURY

We first observed in our type II cell studies that the release of a ^{51}Cr prelabel, known to bind to cytosolic proteins typically of more than 10,000 kD, had a threefold greater endogenous rate of release than LDH. The ^{51}Cr release was stimulated by isoproterenol, which activates surfactant secretion, whereas LDH release was unaffected (Table 3). From this, we assume that ^{51}Cr was associated with an exocytotic event not linked with cell injury and that LDH release was a better marker of oxidant stress and cell injury. Thus, LDH release served as a marker for cell injury for many experiments such as those reported in Figure 9, 10, and 11. In a subsequent study of type II cell responses to in vitro culture and hyperoxia, LDH, DNA, and preincorporated 8-[^{14}C]adenine release were all utilized simultaneously as markers of cell injury. Even though a significant extracellular DNA and adenine release was detected in hyperoxia-exposed mono-

layers, extracellular release of LDH activity was less than would have been expected when compared to the other markers. We then determined that LDH was inactivated by systems that produce $O_2^{\bullet-}$ and H_2O_2 (Figure 4). The strong possibility exists that increased oxidant levels in metabolically stressed or free radical–stressed cultures resulted in LDH inactivation (Freeman and Crapo 1981a; Turrens et al. 1982a,b), thereby decreasing the accuracy of this enzyme as a marker of cellular injury during oxidant stress. The mechanism of inactivation of LDH was not examined, but may be due to oxidation of critical active site sulfhydryl groups (Buchanan and Armstrong 1976). In light of these possibilities we selected the release of 8-[^{14}C]-adenine prelabel as the most reliable and sensitive marker of in vitro oxidant cell injury. This molecule, which is not lipid- or protein-associated upon cell uptake, is not subject to artifact during surfactant secretory processes or oxidative inactivation.

LIPOSOME DELIVERY OF ANTIOXIDANTS TO THE ALVEOLAR EPITHELIUM

Whole lung (Oyarzun et al. 1980) and alveolar epithelial cells (Geiger et al. 1975) take up intratracheally delivered exogenous phospholipids in vivo and reutilize hydrolyzed phospholipids for surfactant lipid synthesis (Jacobs et al. 1983). In vitro, type II alveolar epithelial cells ingest liposomal phospholipids by endocytosis or fusion and degrade internalized lipids (Chander et al. 1983). From these studies, it was unclear whether an enzyme entrapped in the aqueous compartment of liposomes could be incorporated into hydrolytically active type II cells in a catalytically active form. In this study, we observed that an active enzyme could be delivered to type II cells, because catalase- and SOD-specific activity increased up to 20-fold (Table 5) after cell incubation with liposome-entrapped enzymes.

Anionic liposomes, consisting of phosphatidylcholine plus phosphatidylglycerol (to impart the anionic charge), have been shown to be efficiently taken up by type II cells (Chander et al. 1983). We used cationic liposomes consisting of dipalmitoylphosphatidylcholine, cholesterol, and stearylamine because the efficiency of macromolecule capture or entrapment was more favorable using this lipid composition. Also, stearylamine was found to enhance the uptake of liposomes by type II cells. When stearylamine was omitted from liposome preparations, type II cell liposome uptake was reduced, and enzyme capture entrapment efficiency decreased. Thus, net efficiency of enzyme delivery to the isolated cells was reduced dramatically without the addition of a cationic amphiphile.

Unique physical characteristics of catalase are probably responsible for the significant proportion of catalase activity (40%) associated with the extraliposomal surface. Although catalase was succinoylated before use to prevent liposome aggregation (Tanswell and Freeman 1987), it still adhered avidly to liposome surfaces. Liposomal surface–associated catalase has been shown capable of significant H_2O_2 metabolism. In the present study, the finding of cell surface–associated catalase, inferred by a trypsin-sensitive pool of enzyme activity, did not lend insight into the possible mechanisms of liposome uptake. Both endocytosis and fusion involve initial cell-surface association of liposomes. The observations in Figures 8, 9, and 10 suggest that type II cells and potentially other alveolar surface-associated cells, after treatment with catalase-containing liposomes, would be rendered more resistant to H_2O_2 derived from extracellular sources, such as ozone decomposition macrophages or neutrophils. Augmentation of intracellular catalase activity would be expected to enhance resistance of the alveolar epithelium to injury derived from increased production of reactive O_2 species from subcellular sites as well, such as that induced by exposure to hyperoxia or xenobiotics such as paraquat. We conclude it is beneficial that liposome-mediated delivery of antioxidant occurs to both intracellular and cell surface compartments, because cytosolic and membrane components both serve as target sites for oxidant injury.

In the present study, cells treated with liposome-entrapped catalase were more resistant to oxidant-mediated injury than controls. It should be noted that XO generates both $O_2^{\bullet-}$ and H_2O_2, which, in turn, can contribute to elevated rates of $^{\bullet}OH$ production (McCord 1983). Complete protection was observed when cells pretreated with catalase liposomes were exposed to a mixture of glucose and glucose oxidase, which yields H_2O_2 as the only primary reactive oxygen species (Figure 9). Experiments could not be designed to test the protective effect of SOD delivered alone via liposomes, because the substrate of SOD ($O_2^{\bullet-}$) rapidly dismutes spontaneously or by SOD-catalyzed mechanisms yielding H_2O_2, which is toxic in its own right. Thus, catalase liposomes were preferentially used in test systems. Protection against oxidant injury following catalase liposome treatment was found to be dependent on the degree of cellular catalase augmentation. This was demonstrated by the linear relationship between LDH release and type II cell catalase-specific activity (Figure 10). When catalase-specific activity was augmented twofold, only partial protection was obtained in cells having 200 mU/mL glucose oxidase added to the culture medium. Complete protection required at least a fourfold augmentation of catalase-specific activity.

Based on results of in vitro studies, delivery of liposome-encapsulated antioxidants to the lung by intratracheal instillation or aerosolization would be expected to augment epithelial cell defenses and protect against oxidant injury

to the epithelium. This approach has already been shown to improve the survival of rats in hyperoxia (Turrens et al. 1984; Padmanabhan et al. 1985). Targeting the alveolar epithelial surface for liposomal delivery may be preferable to other routes of liposomal administration, because intraperitoneal and intravenous injection require very high concentrations of liposomes for the augmentation of lung antioxidant enzymes. These high concentrations can result in liposomal uptake by tissues other than lung tissue and may potentially involve unwanted side effects. We conclude that liposome-mediated delivery of antioxidant enzymes to the alveolar surface is of therapeutic benefit in lung pathophysiology mediated by oxidants.

LIPOSOME DELIVERY OF THE LIPOPHILIC ANTIOXIDANT, α-TOCOPHEROL

The hydrophobic antioxidant, D,L-α-tocopherol, was readily incorporated into liposome membranes and was delivered to type II cells much more efficiently in a liposome-associated form than when bound to the serum albumin and lipoproteins of culture medium (Tables 7, 8, and 9). Our experiments showed that α-tocopherol could be acutely delivered to alveolar epithelium using liposomes as vectors, with maximal cell uptake occurring in vitro within six hours (Figure 7). Separate studies showed that no apparent toxicity was induced in type II cells by liposomal α-tocopherol combinations. These data show that liposomes can serve as efficient vectors for providing hydrophobic low molecular weight antioxidants, as well as aqueous-soluble macromolecular antioxidant enzymes to target cells at risk for oxidant injury.

IN VIVO DELIVERY OF LIPOSOME-ENTRAPPED SOD AND CATALASE

Our data in Figures 13 through 18 show that pulmonary SOD and catalase activity can be increased significantly in the lungs of animals receiving a single intratracheal instillation of 20 μM liposome phospholipid suspended in 10 mL saline. Peak SOD activities in lung homogenates were observed approximately four hours after liposome instillation and returned to control levels within 24 hours after instillation. In contrast, catalase activities were significantly higher than those of controls 24 hours after liposome instillation. Analysis of the pulmonary spatial distribution of liposome-instilled fluorescent SOD and catalase showed that there were no differences in liposome distribution or antioxidant enzyme uptake among lung lobes. Fluorescence analysis of SOD and catalase uptake showed treated tissues to have fluorescence intensities greater than the autofluorescence observed with both controls and empty liposome-treated lung tissue. The tissue distribution of SOD and catalase was cytosolic and was not limited to alveolar type II cells, with fluorescence observed in pulmonary macrophages and alveolar type II cells. These observations show that it is possible to maximize antioxidant administration to the lung by selective delivery to target tissues. Liposomal antioxidant or drug delivery into the lung can be accomplished by both intratracheal instillation or aerosol administration. Future studies are required to determine which approach is optimal for producing a diffuse and uniform distribution of the delivered dose, with recognition that the percentage of a given aerosolized dose that reaches the airways will be much less than that resulting from intratracheal instillation.

PULMONARY TYPE II CELL OXIDANT GENERATION: ROLE OF XDH/XO AND EFFECTS ON SURFACTANT BIOSYNTHESIS

The XDH/XO activity of freshly isolated rabbit type II cells inhibits endogenous surfactant phospholipid biosynthesis by decreasing the incorporation of palmitate and choline into phosphatidylcholine (Figure 20). This modulatory influence of the XO-derived reactive oxygen species may affect other type II cell functions as well. It has been established that maintenance of type II cells in culture results in enhanced oxidant-induced cytotoxicity, loss of cell antioxidant enzymes, impaired surfactant synthesis, and decreased uptake of polyamines (Figure 2)(Cott et al. 1987; Aerts et al. 1988; Kawada et al. 1988; Kameji et al. 1989). Cellular antioxidant enzymes (i.e., superoxide dismutases, catalase, and various peroxidases) are responsible for enzymatically reducing PROS to H_2O. During culture, an imbalance between cellular antioxidant enzyme–specific activities and PROS formation may account for some of the in vitro dedifferentiation of type II cells.

Protection of cultured type II cells from the detrimental effects of extracellular XO-derived PROS is best afforded by extracellular catalase rather than other antioxidant enzymes (Szoka and Papahadjopoulos 1980; Freeman et al. 1983; Tanswell and Freeman 1987). Similar protection of these cells from intracellular XO-derived reactive oxygen species by intracellular catalase may also occur. Therefore, an interrelationship between type II cell XO activity, catalase activity, and culture-induced changes in these enzyme activities exists. Freshly isolated rat type II cell XDH and XO activity was 33 $\mu U/10^6$ cells. We have shown that XDH/XO has a five-fold greater specific activity for xanthine as substrate compared with pterin (Beckman et al. 1989). Thus, type II cell XDH/XO activity would be 165 $\mu U/10^6$ cells if xanthine were the substrate. If type II cell XDH/XO is completely converted to the oxidase form, XO has the potential

to generate intracellular $O_2^{\cdot-}$ and H_2O_2 at a rate of 33 and 132 pmol/minute per 10^6 cells, respectively. This assumes a proportion of 20% univalent reduction of $O_2^{\cdot-}$ and an 80% divalent reduction of O_2 to H_2O_2 by XO (Fridovich 1970). Cellular XDH/XO activity was also detected in cells the bronchoalveolar lavage, which mainly consisted of macrophages, along with some lymphocytes, polymorphonuclear leukocytes, and red blood cells. These cells were a 16% contaminant in the final type II cell preparation and were calculated to contribute a maximum of 12% of the total XDH/XO activity in freshly isolated type II cells. We believe that many of these cells did not adhere to culture dishes after the initial 36-hour plating period, thus contributing less to the type II cell monolayer XDH/XO activities 35 to 132 hours after plating. Previous investigations have also detected XDH and XO activity associated with macrophages and polymorphonuclear leukocytes isolated from humans and mice. However, the importance of XO-derived PROS to the overall generation of partially reduced oxygen by these cells is controversial and probably minimal (Jones et al. 1985).

Culturing rat type II cells resulted in a specific loss of XDH/XO activity when normalized for either cell protein or DNA. Loss of cellular XDH/XO occurred during a period when cell numbers, determined by DNA/cm^2, were constant, and cell protein content was increasing. The loss of type II cell XDH/XO also occurred when cell LDH activity was stable. Thus, the decrease in XDH/XO was not due to a general loss of cell enzyme and protein content or a reduction in protein synthesis. These data are consistent with other observations of culture-induced alterations in type II cell enzymatic activities (Cott et al. 1987; Kawada et al. 1988).

Purified XDH can be irreversibly converted to XO by various proteolytic enzymes. Reversible conversion of purified XDH to XO can also occur by sulfhydryl oxidation, with XDH regeneration occurring after the addition of sulfhydryl reducing agents. In our investigation, the lysing buffer inhibited artifactual reversible and irreversible XO formation by the addition of a sulfhydryl reducing agent and various antiproteases. Reversible XO in the type II cell lysates was converted to XDH by the dithiothreitol in the lysing buffer. During culture, the percentage of type II cell XO increased from 31% in freshly isolated cells to 67% in cells cultured for 132 hours. These results suggest that increased cellular XO activity during culture did not occur via oxidation of sulfhydryls to disulfides. Other explanations for XO formation from XDH include oxidation of sulfhydryls to sulfenic, sulfinic, and sulfonic acid derivatives, and partial proteolysis of XDH.

Possible cellular mechanisms accounting for the loss of cultured type II cell XDH/XO and antioxidant enzyme activities and the XDH to XO conversion of remaining cell XHD/XO activity are unknown. Inactivation of both cellular XDH/XO and antioxidant enzyme activities by PROS during type II cell culture is possible. Autoinactivation of XO occurs by partially reduced oxygen-mediated mechanisms (Lynch and Fridovich 1979). In contrast, LDH activity, which may also be inactivated by XO-derived PROS (Buchanan and Armstrong 1976), was not decreased in these same cultured cells. The different rates of loss of oxidant-sensitive enzymes may suggest more sensitivity of XDH/XO and antioxidant enzymes to oxidant inactivation, or an alternative mechanism explaining the loss of XDH/XO and antioxidant enzyme activities. For example, decreased enzyme activities may be due to normal turnover mechanisms of enzymes without resynthesis. An organ culture of chick liver resulted in a loss of XDH/XO activity, because an inability to synthesize a functional form of the enzyme (Thompson et al. 1979). The $t_{1/2}$ for the loss of chick XDH/XO activity was 11 hours, which paralleled the loss of type II cell XDH/XO activity in our investigation. Loss of cell XDH/XO and catalase may also be related to other phenotypic alterations that occur after type II cell isolation and culture. Decreased rates of incorporation of precursors for surfactant synthesis, loss of antioxidant enzymes and XDH/XO, as well as the occurrence of other cellular and morphologic changes have been observed in cultured type II cells. Culturing type II cells may result in a loss of cell regulatory mechanisms necessary to maintain XDH/XO, antioxidant enzymes, and other differentiated cell processes.

Recent observations have demonstrated that certain substrata alone or in combination with other modifications to the culture media or serum, may delay type II cell dedifferentiation (Dobbs et al. 1985; Cott et al. 1987; Kawada et al. 1988; Rannels et al. 1989). Modifications of cell substrate include maintenance of type II cells on Englebreth-Holm-Swan mouse sarcoma matrix, human anionic basement membrane, and fibroblast feeder layers supported by floating collagen gels (Wand and Rajagopalan 1976; Szoka and Papahadjopoulos 1980). Furthermore, addition to the culture media of charcoal-stripped rat serum, sarcoma matrix–soluble factors, or hydrocortisone and dibutyryl cyclic adenosine monophosphate (AMP), individually, or in combination with substrata modifications, further delays the dedifferentiation of cultured type II cells. However, these culture modifications still do not maintain stable type II cell morphological and biochemical characteristics.

Whether these culture modifications will also decrease the rate of loss of cell XDH/XO and antioxidant enzyme activities in cultured type II cells is currently unknown. In our investigations, type II cells were cultured on disks pretreated with fibronectin.

SUMMARY

We have demonstrated an ability to modify alveolar epithelial cell antioxidant defenses in vitro and in vivo. Until culture conditions for type II cells improve, both the detrimental effects of oxidants and the protection of type II cells that can be afforded by antioxidants will be difficult to assess in vitro, due to culture-induced changes in the phenotypic expression of these cells. Thus, reactive oxygen metabolism may be best studied in vitro utilizing freshly isolated type II cells, which may not be compatible with long-term pharmacologic studies. Although type II cells may actually differentiate into type I cells in vitro, no data exist regarding type I cell oxidant-producing and antioxidant characteristics to reveal whether our observed type II cell changes during culture are reflective of type I cells.

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REFERENCES

Aerts C, Voisin C, Wallaert B. 1988. Type II alveolar epithelial cell in vitro culture in aerobiosis. Eur Respir J 1:738–747.

Andreoli SP, Baehner RL, Bergstein JM. 1985. In vitro detection of endothelial cell damage using 2-deoxy-D-^{3}H-glucose: Comparison with chromium 51, ^{3}H-leucine, ^{3}H-adenine, and lactate dehydrogenase. J Lab Clin Med 106: 253–261.

Aust SD, Morehouse LA, Thomas CE. 1985. Role of metals in oxygen radical reactions. J Free Radic Biol Med 1:3–25.

Baker RR, Holm BA, Panus PC, Matalon S. 1989. Development of O_2 tolerance in rabbits with no increase in antioxidant enzymes. J Appl Physiol 66:1679–1684.

Beckman JS, Parks DA, Pearson JD, Marshall PA, Freeman BA. 1989. A sensitive fluorometric assay for measuring xanthine dehydrogenase and oxidase in tissues. J Free Radic Biol Med 6:607–615.

Beutler E. 1975. In: Red cell metabolism: A manual of biochemical methods, pp. 71–75. Grune & Stratton, New York, NY.

Blum J, Fridovich I. 1985. Inactivation of glutathione peroxidase by superoxide radical. Arch Biochem Biophys 240:500–508.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Brown LA, Longmore WJ. 1981. Adrenergic and cholinergic regulation of lung surfactant secretion in the isolated perfused rat lung and in the alveolar type II cell in culture. J Biol Chem 256:66–72.

Buchanan JD, Armstrong DA. 1976. Free radical inactivation of lactate dehydrogenase. Int J Radiat Biol 30:115–1127.

Chander A, Claypool WD Jr, Strauss JF 3d, Fisher AB. 1983. Uptake of liposomal phosphatidylcholine by granular pneumocytes in primary culture. Am J Physiol 245:C397–C404.

Cott GR, Walker SR, Mason RJ. 1987. The effect of substratum and serum on the lipid synthesis and morphology of alveolar type II cells in vitro. Exp Lung Res 13:427–447.

Crapo JD, Barry BE, Chang LY, Mercer RR. 1984. Alterations in lung structure caused by inhalation of oxidants. J Toxicol Environ Health 13:301–321.

Crapo JD, Sjostrom K, Drew RT. 1978. Tolerance and cross-tolerance using NO_2 and O_2: I. Toxicology and biochemistry. J Appl Physiol 44:364–369.

Crapo JD, Tierney DF. 1974. Superoxide dismutase and pulmonary oxygen toxicity. Am J Physiol 226:1401–1407.

Crim C, Simon RH. 1988. Effects of oxygen metabolites on rat alveolar type II cell viability and surfactant metabolism. Lab Invest 58:428–437.

Davies KJ. 1986. Intracellular proteolytic systems may function as secondary antioxidant defenses: An hypothesis. J Free Radic Biol Med 2:155–173.

DeLucia AJ, Hoque PM, Mustafa MG, Cross CE. 1972. Ozone interaction with rodent lung: Effect on sulfhydryls and sulfhydryl-containing enzyme activities. J Lab Clin Med 80:559–566.

Dobbs LG, Mason RJ. 1979. Pulmonary alveolar type II cells isolated from rats: Release of phosphatidylcholine in response to beta-adrenergic stimulation. J Clin Invest 63:378–387.

Dobbs LG, Williams MC, Brandt A. 1985. Changes in biochemical characteristics and pattern of lectin binding of al-

veolar type II cells with time in culture. Biochim Biophys Acta 846:155–166.

Douglas JS, Curry G, Geffkin A. 1977. Superoxide dismutase and pulmonary ozone toxicity. Life Sci 20:1187–1192.

Engerson TD, McKelvey TG, Rhyne DB, Boggio EB, Snyder SJ, Jones HP. 1987. Conversion of xanthine dehydrogenase to oxidase in ischemic rat tissues. J Clin Invest 79:1564–1570.

Evans MJ. 1984. Oxidant gases. Environ Health Perspect 55:85–95.

Fiszer-Szafarz B, Szafarz D, Guevara de Murillo A. 1981. A general, fast, and sensitive micromethod for DNA determination application to rat and mouse liver, rat hepatoma, human leukocytes, chicken fibroblasts, and yeast cells. Anal Biochem 110:165–170.

Forman HJ, Fisher AB. 1981. Antioxidant enzymes of rat granular pneumocytes: Constitutive levels and effect of hyperoxia. Lab Invest 45:1–6.

Freeman BA, Crapo JD. 1981. Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. J Biol Chem 256:10986–10992.

Freeman BA, Crapo J. 1982. Biology of disease, free radicals and tissue injury. Lab Invest 47:412–426.

Freeman BA, Mason RJ, Williams MC, Crapo JD. 1986. Antioxidant enzyme activity in alveolar type II cells after exposure of rats to hyperoxia. Exp Lung Res 10:203–222.

Freeman BA, Sharman MC, Mudd JB. 1979. Reaction of ozone with phospholipid vesicles and human erythrocyte ghosts. Arch Biochem Biophys 197:264–272.

Freeman BA, Young SL, Crapo JD. 1983. Liposome-mediated augmentation of superoxide dismutase in endothelial cells prevents oxygen injury. J Biol Chem 258:12534–12542.

Fridovich I. 1970. Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. J Biol Chem 245:4053–4057.

Geiger K, Gallagher ML, Hedley-White J. 1975. Cellular distribution and clearance of aerosolized dipalmitoyl lecithin. J Appl Physiol 39:759–766.

Gilder H, McSherry CK. 1974. Mechanisms of oxygen inhibition of pulmonary surfactant synthesis. Surgery 76:72–79.

Grisham MB, Hernandez LA, Granger DN. 1986. Xanthine oxidase and neutrophil infiltration in intestinal ischemia. Am J Physiol 251:567–574.

Haagsman HP, Schuurmans EA, Alink GM, Batenburg JJ, van Golde LM. 1985. Effects of ozone on phospholipid synthesis by alveolar type II cells isolated from adult rat lung. Exp Lung Res 9:67–84.

Heath RL. 1979. Breakdown of ozone and formation of hydrogen peroxide in aqueous solutions of amine buffers exposed to ozone. Toxicol Lett 4:449–453.

Hodgson EK, Fridovich I. 1975. The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide: Inactivation of the enzyme. Biochemistry 14:5294–5299.

Jackson RM, Frank L. 1984. Ozone-induced tolerance to hyperoxia in rats. Am Rev Respir Dis 129:425–429.

Jacobs H, Jobe A, Ikegami M, Conaway D. 1983. The significance of reutilization of surfactant phosphatidylcholine. J Biol Chem 258:4159–4165.

Jones HP, Grisham MB, Bose SK, Shannon VA, Schott A, McCord JM. 1985. Effect of allopurinol on neutrophil superoxide production, chemotaxis, or degranulation. Biochem Pharmacol 34:3673–3676.

Kameji R, Rannels SR, Pegg AE, Rannels DE. 1989. Spermidine uptake by type II pulmonary epithelial cells in primary culture. Am J Physiol 256:160–167.

Kawada H, Shannon JM, Mason RJ. 1988. Improved maintenance of adult rat alveolar type II cell differentiation in vitro: Effect of hydrocortisone and cyclic AMP. Biochim Biophys Acta 972:152–166.

Kirby TW, Fridovich I. 1982. A picomolar spectrophotometric assay for superoxide dismutase. Anal Biochem 127:435–440.

Kono Y, Fridovich I. 1982. Superoxide radical inhibits catalase. J Biol Chem 257:5751–5754.

Lynch RE, Fridovich I. 1979. Autoinactivation of xanthine oxidase: The role of superoxide radical and hydrogen peroxide. Biochim Biophys Acta 571:195–200.

McCord JM. 1983. The biochemistry and pathophysiology of superoxide. Physiologist 26:156–158.

McCord JM. 1985. Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med 312:159–163.

Menzel DB. 1976. Nitrogen oxides and ozone: The role of free radicals in the toxicity of air pollutants. In: Free Radicals in Biology, Vol II (Pryor WA, ed.) pp. 181–202. Academic Press, Orlando, FL.

Mudd JB, Freeman BA. 1977. Reaction of Ozone with Biological Membranes. In: Biochemical Effects of Environmental Pollutants (Lee SD, ed.) pp. 97–34. Ann Arbor Science Publishers, Ann Arbor, MI.

Oyarzun MJ, Clements JA, Baritussio A. 1980. Ventilation enhances pulmonary alveolar clearance of radioactive dipalmitoyl phosphatidylcholine in liposomes. Am Rev Respir Dis 121:709–721.

Padmanabhan RV, Gudapaty R, Liener IE, Schwartz BA, Hoidal JR. 1985. Protection against pulmonary oxygen toxicity in rats by the intratracheal administration of liposome-encapsulated superoxide dismutase or catalase. Am Rev Respir Dis 132:164–167.

Panus PC, Freeman BA. 1988. Liposome-entrapped superoxide dismutase: In vitro and in vivo effects. In: Liposomes as Drug Carriers (Gregoriadis G, ed.) pp. 473–482. Wiley & Sons, New York, NY.

Panus PC, Shearer J, Freeman BA. 1988. Pulmonary metabolism of reactive oxygen species. Exp Lung Res 14:959–976.

Parks DA, Williams TK, Beckman JS. 1988. Conversion of xanthine dehydrogenase to oxidase in ischemic rat intestine: A re-evaluation. Am J Physiol 254:768–774.

Prutz WA, Monig H, Butler J, Land EJ. 1985. Reactions of nitrogen dioxide in aqueous model systems: Oxidation of tyrosine units in peptides and proteins. Arch Biochem Biophys 243:125–134.

Rannels SR, Grove RN, Rannels DE. 1989. Matrix-derived soluble components influence type II pneumocytes in primary culture. Am J Physiol 256:621–629.

Rietjens IM, Van Bree L, Marra M, Poelen M, Rombout J, Alink GM. 1985. Glutathione pathway enzyme activities and the ozone sensitivity of lung cell populations derived from ozone exposed rats. Toxicology 37:205–214.

Rodell TC, Cheronis JC, Ohnemus CL, Piermattei DJ, Repine JE. 1987. Xanthine oxidase mediates elastase-induced injury to isolated lungs and endothelium. J Appl Physiol 63:2159–2163.

Szoka F Jr, Papahadjopoulos D. 1980. Comparative properties and methods of preparation of lipid vesicles. Annu Rev Biophys Bioeng 9:467–508.

Tanswell AK, Freeman BA. 1987. Liposome-entrapped antioxidant enzymes prevent lethal O_2 toxicity in the newborn rat. J Appl Physiol 63:347–352.

Terada LS, Beehler CJ, Banerjee A, Brown JM, Grosso MA, Harken AH, McCord JM, Repine JE. 1988. Hyperoxia and self- or neutrophil-generated O_2 metabolites inactivate xanthine oxidase. J Appl Physiol 65:2349–2353.

Thompson JM, Nickels JS, Fishe JR. 1979. Synthesis and degradation of xanthine dehydrogenase in chick liver. Biochim Biophys Acta 568:157–176.

Turrens JF, Crapo JD, Freeman BA. 1984. Protection against oxygen toxicity by intravenous injection of liposome-entrapped catalase and superoxide dismutase. J Clin Invest 73:87–95.

Turrens JF, Freeman BA, Crapo JD. 1982a. Hyperoxia increases H_2O_2 release by lung mitochondria and microsomes. Arch Biochem Biophys 217:411–421.

Turrens JF, Freeman BA, Levitt JG, Crapo JD. 1982b. The effect of hyperoxia on superoxide production by lung submitochondrial particles. Arch Biochem Biophys 217:401–410.

Waud WR, Rajagopalan KV. 1976. The mechanism of conversion of rat liver xanthine dehydrogenase from an NAD+-dependent form (type D) to an O_2-dependent form (type O). Arch Biochem Biophys 172:365–379.

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PUBLICATIONS RESULTING FROM THIS RESEARCH

Fridovich I, Freeman BA. 1986. Antioxidant defenses in the lung. Annu Rev Physiol 48:693–702.

Buckley BJ, Tanswell AK, Freeman BA. 1987. Liposome mediated augmentation of catalase in type II alveolar epithelial cells protects against hydrogen peroxide injury. J Appl Physiol 63:359–367.

Tanswell AK, Freeman BA. 1987. Liposome-entrapped antioxidant enzymes prevent lethal O_2 toxicity in the newborn rat. J Appl Physiol 63:347–352.

Panus PC, Freeman BA. 1988. Liposome-entrapped superoxide dismutase: In vitro and in vivo effects. In: Liposomes as Drug Carriers (Gregoriadis G, ed.) pp. 473–482. Wiley & Sons, New York, NY.

Panus PC, Shearer J, Freeman BA. 1988. Pulmonary metabolism of reactive oxygen species. Exp Lung Res 14:959–976.

Beckman JS, Parks DA, Pearson J, Marshall P, Freeman BA. 1989. A sensitive fluorometric assay for measuring xanthine dehydrogenase and oxidase in tissues. Free Radic Biol Med 6:607–615.

Matalon SM, Beckman JS, Duffey ME, Freeman BA. 1989. Oxidant inhibition of epithelial active sodium transport. Free Radic Biol Med 6:557–564.

Panus PJ, Matalon SM, Freeman BA. 1989. Responses of type II pneumocyte antioxidant enzymes to normoxic and hyperoxic culture. In Vitro Cell Dev Biol 25:821–829.

Baker RR, Panus PC, Holm BA, Engstrom PC, Freeman BA, Matalon A. 1990. Endogenous xanthine oxidase–derived O_2 metabolites inhibit surfactant metabolism. Am J Physiol 258:328–334.

Matalon S, Holm BA, Baker RR, Whitfield MK, Freeman BA. 1990. Characterization of antioxidant activities of pulmonary surfactant mixtures. Biochim Biophys Acta 1035: 121–127.

Panus PJ, Freeman BA. 1990. Responses of type II pneumocytes to oxidant stress. In: Biology, Toxicology, and Carcinogenesis of Respiratory Epithelium (Thomasson D, Nettesheim P, eds.) pp. 129–144. Hemisphere Publishing Corp., New York, NY.

Tanswell AK, Olson DM, Freeman BA. 1990. Liposome-mediated augmentation of antioxidant defenses in fetal rat pneumocytes. Am J Physiol 258:165–172.

Panus PC, Burgess B, Freeman BA. 1991. Characterization of type II alveolar epithelial cell xanthine oxidase. Biochim Biophys Acta 1091:303–309.

ABBREVIATIONS

AMP	adenosine monophosphate
AOE	antioxidant enzyme
CHAPS	3-[(3-chloramidopropyl)dimethylamino]--propane sulfonate
CuZn	copper zinc
DPPC	dipalmitoylphosphatidylcholine
GSH Px	cytosolic glutathione peroxidase
FITC	fluorescein isothiocyanate
H_2O	water
H_2O_2	hydrogen peroxide
KOH	potassium hydroxide
LDH	lactate dehydrogenase
Mn	manganese
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NO_2	nitrogen dioxide
O_2	oxygen
$O_2^{\cdot-}$	superoxide

O_3	ozone
•OH	hydroxyl radical
ppm	parts per million
PROS	partially reduced oxygen species
SOD	superoxide dismutase
XO	xanthine oxidase
XDH	xanthine dehydrogenase

INTRODUCTION

A Request for Applications (RFA 84–3) that solicited proposals for "Mechanisms of Oxidant Toxicity" was issued by the Health Effects Institute (HEI) in the summer of 1984. In response to this RFA, Dr. Bruce A. Freeman from Duke University Medical Center in Durham, NC submitted a proposal to HEI, entitled "Oxidant Injury to the Alveolar Epithelium: Biochemical and Pharmacologic Studies." Before beginning his study, Dr. Freeman changed his affiliation to the University of Alabama at Birmingham. The three-year project began in October 1985, and total expenditures were $373,630. The Investigators' Report was received for review at HEI in January 1991 and a revised report was accepted by the Health Review Committee in December 1991. During the review of the Investigators' Report, the Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary. The Health Review Committee's Commentary is intended to place the Investigators' Report in perspective, as an aid to the sponsors of HEI and to the public.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA) sets standards for oxidants (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1990. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Sections 202(a), (b)(1), (g), and (h) and sections 207(c)(4), (5), and (6) impose specific requirements for reducing motor vehicle emissions of certain oxidants (and other pollutants), and, in some cases, provide the EPA with limited discretion to modify those requirements.

In addition, Section 109 of the Clean Air Act provides for the establishment of National Ambient Air Quality Standards (NAAQS) to protect the public health. The current primary and secondary NAAQS for ozone is 0.12 parts per million (ppm).* This standard is met when the number of days per year with maximum hourly average concentrations above 0.12 ppm is equal to or less than one. Section 181 of the Act classifies the 1989 nonattainment areas according to the degree that they exceed the NAAQS and assigns a primary standard attainment date for each classification.

Determining appropriate standards for emissions of oxidants and their precursors depends, in part, on an assessment of the health risks that they present. Thus, research on the health effects of ozone in studies like this one is essential to the informed regulatory decision-making required by the Clean Air Act.

SCIENTIFIC BACKGROUND

Ozone and nitrogen oxides are significant contributors to the photochemical smog that exists in many areas of the United States (U.S. Environmental Protection Agency 1991). Humans who exercise and inhale these gases for short periods can experience alterations in breathing patterns (Lippmann 1989; Samet and Utell 1990). The adverse human health effects caused by repeated inhalation of these gases over the course of a lifetime are not known. However, structural alterations observed in the lungs of animals after long-term inhalation exposures indicate that oxidant gases may cause or contribute to the development of chronic lung disease (Chang et al. 1986; Lippman 1989).

Ozone and nitrogen oxides are termed oxidant gases because they act as oxidizing agents that attract electrons from other molecules within cells and tissues. When inhaled, these gases can react chemically with a variety of lung components in ways that alter these components and potentially damage the lungs. Precursors for these gases are emitted into the ambient air by motor vehicles and stationary sources because of incomplete fuel combustion. These precursors undergo chemical reactions in the atmosphere with hydrocarbons and sunlight, and, as a result, they generate ozone and nitrogen dioxide. Because of the reported adverse health effects associated with inhaling these oxidant gases, the EPA (1982, 1986) has established NAAQS levels for both ozone and nitrogen dioxide. Although the NAAQS for nitrogen oxides generally has been met during the last decade (U.S. Environmental Protection Agency 1991), levels exceeding the ozone standard continue (National Research Council 1992). The EPA (1991) reported that in 1990 approximately 25% of the population in the United States lived in areas in which the ozone standard was exceeded.

THE ALVEOLAR EPITHELIUM AS A TARGET OF OXIDANT INJURY

The gas exchange (alveolar) region of the lungs provides a large target area for the injury that can be produced by in-

* A list of abbreviations appears at the end of the Investigators' Report for your reference.

haling oxidant gases (Weibel 1984). As a basis for comparison, the total surface area of an average human's lungs is slightly less than that of a tennis court. Alveolar type I and type II epithelial cells are the two principal cells that form the interface between air and tissue in the lungs. Alveolar type I epithelial cells are very thin, flat cells that line approximately 97% of the alveolar surface. Alveolar type II epithelial cells are cuboidal cells that produce surfactant, a substance essential for keeping open the small airspaces (alveoli) in the lungs. Alveolar type II epithelial cells also serve as the progenitors for both type II and type I cells during normal cell turnover and after epithelial injury.

Damage to alveolar epithelial cells and other cells in the lungs caused by oxidant gases has been attributed to generation of an excess of reactive oxygen molecules, including superoxide anions ($O_2^{\bullet-}$), hydroxyl radicals ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) (Mustafa and Tierney 1978). Inhaled oxidant gases interact with polyunsaturated fatty acids in cell membranes and other cell components to produce chemical reactions that lead to the generation of these reactive oxygen molecules. These molecules then can initiate chemical chain reactions that produce more reactive oxygen molecules. Reactive oxygen molecules have the potential to damage a variety of cellular components, including enzymes, genetic material, and structural proteins; such events can lead to cell injury and, potentially, cell death (Freeman and Crapo 1982).

Normal cellular metabolism requires oxygen and continually produces reactive oxygen molecules in small quantities. However, cells have antioxidant defenses that protect them against the oxidizing effects of these molecules. These defenses include antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, as well as an antioxidant located in cell membranes, α-tocopherol (vitamin E). Antioxidants protect the cells by converting reactive oxygen molecules to less reactive or nontoxic compounds. For a more extensive review of reactive oxygen molecules and antioxidant defenses, the reader is directed to the excellent discussion provided by the investigators in the Introduction section of their report.

Environmental exposures to oxidant gases, particularly ozone, present a health risk to large segments of the United States population because they may increase the oxidant burden in the lungs (Crapo et al. 1992). The extent of this oxidant burden is directly related to the levels of reactive oxygen molecules produced by inhaling these gases. Exposure to hyperoxia, that is, concentrations of oxygen that exceed the usual 20% present in the air we breathe, also can produce excessive levels of reactive oxygen molecules. As a result, hyperoxic exposures can serve as a useful model for oxidant injury produced by environmental oxidant gases. Such exposures often are used clinically to increase oxygen delivery in patients who have compromised lung function. However, these exposures require careful monitoring of the oxygen concentrations to insure that the beneficial effects of this therapeutic approach are not outweighed by the lung damage caused by an increased oxidant burden.

Results from animal experiments and human clinical studies with oxidant gases demonstrate that the acute damage is evidenced by cell damage, leakage of blood components into the alveolar spaces (edema), and an influx of inflammatory cells (Morrow 1984; Crapo 1986; Lippmann 1989). Although the extent of this damage depends significantly on the specific oxidant gas, its concentration, the duration of exposure, and the level of exercise (the combination of which can be used to estimate dose), a similar pattern of cell injury, edema, and inflammatory cell influx results. The inflammatory cell influx actually can add to the existing oxidant burden caused by the gas exposure because these cells produce reactive oxygen molecules as part of their own defensive armament. One outcome of this cumulative oxidant injury may be altered lung structure and function due to fibrotic areas, or scars, formed during tissue repair. An important human health issue is whether the injury and inflammation caused by repeated exposure to oxidant gases in the ambient air contributes to the development of chronic lung disease. Therefore, determining the mechanisms of oxidant injury in lung tissues and the mechanisms that normally protect against it are important for understanding the role of oxidant injury in human disease.

LIPOSOMES AS VEHICLES FOR DELIVERY OF ANTIOXIDANT ENZYMES

One strategy for reducing the damage caused by exposures to oxidant gases and for understanding mechanisms of oxidant tissue injury is to increase the levels of antioxidants in the lungs. As stated previously, without adequate antioxidant protection, an oxidant stress can overwhelm the lungs' protective mechanisms and damage tissues. One simple method of increasing antioxidant levels in the body is by adding them to the diet. Although evidence indicates that animals fed diets deficient in specific antioxidants, such as vitamin E, may exhibit more oxidant-induced injury than normally fed animals, the benefits of supplementing diets with vitamin E levels exceeding those that provide adequate protection have not yet been demonstrated (Pryor 1991). Efforts to protect rats against hyperoxic lung injury by direct delivery of antioxidant enzymes either by aerosol inhalation or by injection into the blood have met with little or no success (Crapo et al. 1977; Turrens et al. 1984). These failures are probably the result of inefficient or inadequate delivery of antioxidants to the sites of toxicity. Because the concept of supplementing the lungs with antioxidants to protect against oxidant damage presents intriguing potential, new ideas are needed to improve delivery efficiency.

During the last decade, liposomes have emerged as a feasible therapeutic approach for delivering drugs and other compounds directly to specific cells and organs in the body, such as the lungs (Cullis et al. 1987). Simply described, liposomes are very small membranes made up of lipids. These membranes can be prepared in the laboratory so that they contain specific compounds or drugs that one wishes to deliver to cells. Although various techniques are available for preparing liposomes, one common preparation method is rehydration of a lipid film that has been dried onto the inner surface of a container. To prepare liposomes, the container lined with the dried lipid film is filled with the desired compound and then sonicated. During sonication, sections of the lipid layer lift off from the wall of the container and encapsulate the desired compound, forming liposomes. Both the lipid composition and final size of the liposomes can be modified during preparation so that the best combination for facilitating liposomal uptake by the target cells can be determined.

Liposomes deliver their contents to targeted cells either by direct fusion with the cell membrane or by phagocytosis of entire liposomes by the cells and subsequent breakdown of the lipid layer. For studies with cells in culture, liposomes can be delivered easily by simply adding them to the cell media. For studies with whole animals, several routes of delivery are available. Liposomes can be delivered to many organs of the body by injecting them into the blood stream. For studies with lungs, liposomes can be delivered directly through inhalation of an aerosol or by instilling them into the trachea. Direct delivery to the lungs by either of these routes is preferable to blood injection because the efficiency of liposome delivery can be reduced when the blood filters through the liver and spleen (Turrens et al. 1984). In addition, inhalation and instillation deliver liposomes directly to the epithelial cells, rather than requiring them to pass through the lung capillaries.

Determining the molecular mechanisms of oxidant injury in lung tissues is important for understanding their potential role in human disease. As noted, the extent to which lung injury caused by repeated exposures to environmental oxidant gases initiates or enhances the pathogenesis of lung disease remains unknown. Studies concerning lung antioxidants can contribute to our knowledge of oxidant injury and the advantages provided by these protective mechanisms.

JUSTIFICATION FOR THE STUDY

Through RFA 84–3, HEI wanted to support studies to understand the relationship between lung injury induced by the inhalation of oxidants and the development of chronic lung disease. Two main objectives listed in this RFA were: (1) to investigate the mechanisms of molecular, cellular, and tissue injury that may be caused by oxidants from motor vehicle emissions; and (2) to develop more sensitive methods for detecting critical targets of oxidant exposure and the associated biologic effects.

Dr. Freeman and his colleagues proposed to evaluate whether they could supplement the levels of antioxidants in the alveolar epithelial cells of the lungs with liposomes containing selected antioxidants. They also wanted to test whether this antioxidant supplementation would modify the responses of the epithelial cells to exposures to hyperoxia or ozone. They proposed to use physiological and biochemical assays to evaluate the effectiveness of antioxidant supplementation to protect against in vitro and in vivo oxidant exposures.

The HEI Research Committee was enthusiastic about the high caliber of the principle investigator and his collaborators. The Committee suggested that Dr. Freeman include in vivo exposures to low concentrations of ozone for periods up to one week. The Committee subsequently recommended funding for a revised proposal in March 1985.

SPECIFIC AIMS AND STUDY DESIGN

Dr. Freeman and his colleagues proposed three specific aims for their study. The first was to devise preparations of liposomes containing the antioxidant enzymes superoxide dismutase and catalase. They would also prepare liposomes with and without α-tocopherol incorporated into the liposome walls. The lipid composition of the liposomes would be varied and then tested to determine the combination that promoted the most efficient liposomal uptake by the alveolar epithelial cells in vitro and in vivo.

The investigators' second specific aim was to evaluate, both in vitro and in vivo, the effectiveness of delivering antioxidants to type II epithelial cells using their liposomal preparations. For the in vitro studies, the investigators would isolate type II epithelial cells from the lungs of normal rats. They would place these cells in culture and add liposomes prepared with lipids labeled with fluorescein and containing the selected antioxidant enzymes. Liposomal binding by the cells and subsequent uptake of liposomal contents would be evaluated by looking for the presence of the fluorescein marker in the cultured cells. For the in vivo studies, superoxide dismutase or catalase would be delivered to rats by having them inhale an aerosol of liposomes containing these enzymes. As in the in vitro studies, identification of fluorescein-labeled lipids in the liposomes would be used to evaluate the uptake of the aerosolized liposomes in the lungs. Antibodies to superoxide dismutase and immunocytochemical methods also would be used to detect this enzyme in the lung tissue with electron

microscopy. Type II epithelial cells also would be isolated from the lungs of rats that had been exposed to the liposome aerosol and would be assayed for their contents of superoxide dismutase, catalase, glutathione peroxidase, and α-tocopherol.

The investigators' third specific aim was to evaluate the effectiveness of antioxidant supplementation in modifying the responses to exposure to hyperoxia and ozone. For the in vitro studies, isolated type II epithelial cells that had been treated with liposomes would be exposed either to 0%, 5%, 20%, 60%, 85%, or more than 95% oxygen at normobaric pressures, or to 0.05, 0.1, 0.5, or 1.0 ppm ozone. For the in vivo studies, rats that had been treated with an aerosol of liposomes would be exposed to 100% oxygen or to 0.1 or 0.25 ppm ozone for up to seven days. Biochemical and physiologic indices would be used to evaluate the extent of injury. Measurements of cell injury would include cell viability, lipid oxidation, glutathione oxidation, and leakage of components from cells.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The investigators successfully completed their first specific aim. They devised liposomes that delivered antioxidant enzymes to type II epithelial cells in vitro and to the alveolar epithelium of rat lungs in vivo.

Completion of the second and third specific aims was compromised by early results from the in vitro studies evaluating baseline levels of antioxidant enzyme levels in type II epithelial cells. The investigators observed that the isolated type II epithelial cells lost their capacity to produce antioxidant enzymes after being in culture from several hours to a few days. Based on these results, the investigators reasoned that the responses of cells exposed in vitro to oxidant gases would not accurately reflect the responses of type II epithelial cells in intact animals to similar oxidant exposures. Although results from in vitro experiments with liposome supplementation could be compared with their matched controls, these results would not correlate with the results from similar manipulations in vivo. This absence of comparable in vitro and in vivo models was the basis for the decision to curtail many of the proposed in vivo hyperoxic studies for testing the effects of antioxidant supplementation with liposomes and to forego the proposed ozone studies.

The investigators chose to alter the focus of their project and study the activity of the enzyme xanthine oxidase as a potential intracellular source of reactive oxygen molecules. Due to this shift in project focus, the results presented in this report differ substantially from those expected from the specific aims presented in the original proposal. These results do, however, add to our understanding of mechanisms of oxidant injury in alveolar epithelial cells and of intracellular sources of reactive oxygen molecules in these cells.

METHODS AND STUDY DESIGN

This study utilized a variety of biochemical and cellular approaches to explore the mechanisms of hyperoxic damage in alveolar type II epithelial cells. The investigators carefully evaluated how hyperoxic exposure affected these cells and their constitutive levels of antioxidant enzymes. The investigators also determined the effectiveness of increasing these constitutive levels by exogenously administering antioxidants with liposomes.

The investigators did not anticipate that the loss of characteristic functions by alveolar type II epithelial cells in culture, termed dedifferentiation, would affect the outcome of their in vitro studies. This dedifferentiation phenomenon, however, has been reported previously for these cells as well as other types of cells in culture (Mason and Dobbs 1980; Dobbs et al. 1985; Whitsett et al. 1985). A pilot study to establish baseline levels of antioxidant enzymes for the duration of the proposed in vitro oxidant exposures might have altered the investigators' experimental design at the outset of their study. Several investigators have reported recently that special culture conditions can support the continued expression of alveolar type II epithelial cell characteristics (Shannon et al. 1992). Although it is pure speculation at this point in time, such growth conditions might have allowed continuation of the investigators' proposed in vitro studies with alveolar type II epithelial cells. However, such experiments are relatively complicated and could interfere with the assessment of cell-related production of or reactions with reactive oxygen molecules.

Interpretation of the data was seriously complicated by changing the animal model used as a source of type II cells for the in vitro studies. In the original proposal, rats were designated as the only animal model for all of the in vitro and in vivo studies. However, due to low yields of numbers of type II cells isolated from rat lungs, the investigators switched to rabbits as a source of these cells for the in vitro studies. This factor complicated subsequent comparisons between data from the in vitro studies using rabbit cells and data from the in vivo studies using rats. As noted by the authors in their Introduction, the pulmonary antioxidant defenses of intact rats and rabbits respond differently to hyperoxic exposures. Therefore, the authors could have presented a more extensive discussion of the similarities and differences between type II cells in culture for these

two species and the impact of any potential differences on the interpretation of their data.

STATISTICAL METHODS

Although the investigators did not provide an overall statistical design for their studies, one was not needed. The proper role of statistical analysis in this report was to provide firm quantitative support for the conclusions drawn from each group of experiments. The investigators linked the results of these analyses to interpret their data.

The appropriate methods for statistical analysis varied from one experimental series to another. These methods are specified in the text, figure legends, and table footnotes. Analysis of variance, which was cited in several instances, is the correct procedure for comparing a continuous endpoint among several groups. When an analysis of variance indicates significant variation among groups, it is usually followed by a specific method such as Duncan's multiple-comparison procedure for comparing pairs of groups, which was also cited repeatedly in these studies. However, when the groups have a factorial structure, Duncan's procedure can be awkward and difficult to interpret. For example, Figure 16 presents data for superoxide dismutase activity from five lung lobes at seven time points, yielding a total of 35 test groups. In this case, inferences could have been expressed more efficiently in terms of the main factors, that is, the lobes and time points.

Most of the data in this report were presented as means with standard errors. This approach provides the reader with a useful index of precision and facilitates quick, informal statistical comparisons that corroborate the authors' conclusions. One deficiency in this regard was the comparison among regression lines in Figure 5. In this figure, the slopes were reported without standard error bars or any other methodological detail to support the claim of statistically significant differences among the regression lines.

Overall, the investigators' choices of statistical tests were appropriate. One notable exception was the use of a one-tailed test in Figure 20. The authors did not state that the xanthine oxidase treatment could only have increased incorporation of palmitate rather than decreasing it. The alternative hypothesis is, therefore, two-sided; the test should be two-tailed like the other tests in Figure 20 and every other test in the report.

RESULTS AND INTERPRETATION

The investigators presented a number of new observations concerning the responses of alveolar type II epithelial cells to hyperoxia. They also demonstrated the feasibility of supplementing the antioxidant capacity of these cells with enzyme-containing liposomes to protect against oxidant damage.

The investigators completed their first specific aim to devise liposomes that would be effective vectors for delivering antioxidant enzymes to type II epithelial cells in vitro and to rat lungs in vivo. They varied the lipid composition of their liposome preparations and determined that positively charged (cationic) liposomes worked best for their experiments. Although other investigators have reported the efficacy of using cationic lipids to promote cell and liposome fusion (Gabizon and Papahadjopoulos 1992), the present investigators are the first to demonstrate this effect for alveolar epithelial cells.

During studies related to their second specific aim, the investigators made a pivotal observation that subsequently altered the course of their project. To determine the responses of alveolar type II epithelial cells to exposures to air and hyperoxia in vitro, the investigators completed a series of experiments to assess the antioxidant enzyme levels in the cells during culture under both conditions. These experiments were to serve as groundwork for later studies to evaluate the effects of antioxidant supplementation on the cells' responses to hyperoxic and ozone exposures. They observed that alveolar type II epithelial cells in culture rapidly lost their capacity to produce antioxidant enzymes during four days of exposure to either air or hyperoxia. As a result of these experiments, the investigators determined that their in vitro epithelial cell model would not accurately reflect events occurring in alveolar epithelial cells in vivo.

The mechanisms responsible for dedifferentiation of alveolar type II epithelial cells in vitro are not known. The published literature on alveolar type II epithelial cells indicates that, once in culture under standard in vitro conditions, these cells rapidly stop expressing many of their characteristic differentiated features (Mason and Dobbs 1980; Dobbs et al. 1985; Whitsett et al. 1985). It is likely that an absence of usual interactions with other cells and substrata, the composition of the growth media, and other unrecognized factors lead to the shutdown (down-regulation) of the expression of genetic message for and synthesis of antioxidant enzymes. The loss of synthesis of messenger RNAs for other cell products, such as surfactant-associated proteins and specific cell surface markers, has been reported for these cells (Shannon et al. 1990).

An important finding of this study was that the antioxidant capacity of alveolar type II epithelial cells could be successfully supplemented in vitro with liposomes. The amounts of superoxide dismutase and catalase both within the cells and bound to the cell surfaces increased 10- to 20-fold by treating cells with liposomes containing these antioxidant enzymes. The amount of catalase that reached the inside of the cells increased the baseline level more than

twofold and protected the cells against subsequent challenge with hyperoxia or with reactive oxygen molecules generated in vitro. The catalase retained its activity during the procedure required to encapsulate it in liposomes; although inactivation of a foreign protein by the cells could have been expected, this did not occur under in vitro conditions but was observed in vivo (Figure 15). Further experiments demonstrated that treating the cells with liposomes containing the antioxidant α-tocopherol in the liposomal layers could augment the cellular levels of this antioxidant up to 20-fold.

The investigators also completed a series of experiments to examine the protective effects of antioxidant supplementation against oxidant stress in vitro. They treated alveolar type II epithelial cells with liposomes containing catalase and then added the enzyme xanthine oxidase or glucose oxidase to the media. After addition of the appropriate substrates, these enzymes produced an oxidant stress to the cells by generating reactive oxygen molecules in the culture media. The investigators then assayed the media for lactate dehydrogenase, an enzyme that is used as a marker of cell injury and death, and found that the antioxidant supplementation reduced the release of this cytotoxicity marker into the media.

The investigators completed several experiments to evaluate the distribution of liposomes containing superoxide dismutase and catalase in the lungs of intact rabbits. Rather than delivering the liposomes via an aerosol, as originally proposed, they chose to instill the liposomes via the trachea. They showed that a single instillation of liposomes resulted in the distribution of the enzymes to all lung lobes. Subsequent measurements of enzyme activities in the lung lobes indicated variations in superoxide dismutase and catalase levels over a three-day observation period. Exposure of these liposome-instilled rats to 100% oxygen produced decreases in the activities of both enzymes compared with rats exposed to room air. These results provided useful supporting data for the feasibility of this delivery route for humans. The potential for extending these findings to clinical studies with human subjects will depend on the results from further extensive testing with cell and animal models.

The investigators also reported findings from several interesting experiments that were not included in the original proposal. They found that hyperoxic exposure reduced the activity of lactate dehydrogenase, and, therefore, caused inaccurate measurement of this enzyme in cytotoxicity assays. This finding is important for other investigators who may also use lactate dehydrogenase as an indicator of oxidant injury. As a result of this finding, the investigators performed experiments to determine a more reliable indicator of cytotoxicity and subsequently selected radiolabeled adenine. Although the inactivation of lactate dehydrogenase was an insightful observation by the investigators, the mid-course change in a key analytical assay complicated the comparison of data from different experiments.

Another important observation was that the activity of xanthine oxidase, an enzyme within alveolar type II epithelial cells, may be a source of reactive oxygen molecules. The investigators proposed that levels of this enzyme could increase within cells during an oxidant exposure because of thiol oxidation or partial proteolysis of xanthine dehydrogenase and its conversion to xanthine oxidase. In vitro experiments indicated that although the combined activity of xanthine dehydrogenase and xanthine oxidase decreased in alveolar type II cells during culture in air, the percentage of activity resulting from xanthine oxidase increased. This reaction could increase intracellular levels of reactive oxygen molecules and account for some oxidative injury to the cells.

The investigators also reported a potential inhibitory effect of oxidants on the production of surfactant. This lipid-protein product of alveolar type II cells lowers surface tension in the alveoli and stabilizes these small air spaces so that they do not collapse. The investigators showed that the addition of xanthine oxidase to the media of cultured alveolar type II cells and the resulting increase in extracellular reactive oxygen molecules decreased the incorporation of precursors needed for surfactant production. Additional experiments demonstrated that the addition of allopurinol, an inhibitor of xanthine oxidase, to these cultures reversed the inhibition of precursor uptake. This finding illustrates the importance of determining the effects of oxidant exposures on the normal metabolic functions of alveolar epithelial cells. It remains unclear whether surfactant synthesis is modified in humans by therapeutic treatment with xanthine oxidase inhibitors, such as the use of allopurinol for gout. However, no clinical effects of this drug on breathing have been reported.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

This study presented interesting findings on the effects of hyperoxia on alveolar type II epithelial cells. With regard to the specific aims proposed originally, many questions related to the effects of ozone exposure on these cells have been left unanswered. Interesting comparisons might have been drawn between the responses of isolated alveolar type II epithelial cells and the responses of whole animals to ozone and hyperoxia. Other unanswered questions include how ozone exposure may have altered the antioxidant en-

zyme levels and how these effects could have been modified by supplementation with liposomes containing antioxidants. Questions also remain regarding the effects of ozone exposure on the cells compared with the effects of a hyperoxic exposure, and regarding whether antioxidant supplementation would have produced comparable benefits.

The investigators considered the dedifferentiation of their isolated alveolar type II epithelial cells in vitro as a significant drawback for subsequent comparisons with alveolar type II cells in vivo. However, this drawback may yet have a positive application. Previous investigators have reported that the dedifferentiation observed in alveolar type II epithelial cells is followed by the expression of many or all of the known markers for alveolar type I epithelial cells, which are the normal progeny of alveolar type II cells in the lungs (Dobbs et al. 1988; Donato et al. 1992). One hypothesis is that alveolar type I cells are exceptionally vulnerable to oxidants because they lose their ability to express the genetic message for antioxidants; synthesis of these important enzymes is, therefore, lost. This hypothesis has not been tested directly because good methods for isolating pure and viable populations of alveolar type I cells are unavailable. The in vitro model of these investigators might provide a system that could be used to test this hypothesis.

The same in vitro system could also be used as a model for studying the mechanisms that regulate transcription and translation of genetic messages for antioxidant enzymes in alveolar epithelial cells. Because inhalation of the oxidant gases in polluted air, such as ozone and nitrogen dioxide, can place an oxidant stress on the lungs, the regulation of genes for antioxidant enzymes is an important area for future research. As shown by Shannon and colleagues (1992), many gene products of isolated alveolar type II cells can be reexpressed in culture if the cells are placed on a complex culture substratum. Such a culture system could provide an interesting venue for exploring molecular mechanisms for regulating key antioxidant genes in alveolar type II epithelial cells.

The results of these studies present other questions regarding the feasibility of antioxidant supplementation for modifying antioxidant defenses in the alveolar epithelial cells. Questions remain regarding the longevity of the antioxidant supplementation to protect against oxidant injury and the maximal amounts of enzyme that can be delivered to the cells. Also unknown is how the cell disposes of the exogenous enzyme and what effects this exogenous supplementation may have on the synthesis and turnover of the cells' own antioxidant enzymes. These questions are pertinent to the use of such protective therapy in animal models of oxidative injury, as well as its protective potential for humans exposed to oxidants. Much more experimental information will be required to determine the long-range usefulness of this potential treatment for humans.

CONCLUSIONS

Understanding the mechanisms of toxicity caused by oxidant gases in polluted air and their implications for human lung disease is important for establishing relevant ambient air quality standards for these pollutants. This study presents new and innovative findings related to the effects of hyperoxic exposures on alveolar epithelial cells.

These experiments were designed to determine whether the antioxidant defenses of alveolar epithelial cells could be supplemented by delivering antioxidant enzymes and α-tocopherol to the cells with liposomes. Another objective was to evaluate whether this supplementation would protect the alveolar cells against oxidant damage caused by exposure to hyperoxia or ozone. As they discuss in their report, the investigators did not have a cell culture system available that supported steady-state expression of antioxidant enzymes in isolated alveolar type II epithelial cells. Because the cells lost many of their key differentiated features, including the ability to produce antioxidant enzymes, the investigators decided to curtail their proposed experiments and addressed the effects of hyperoxia only; no ozone exposures were performed.

These studies did, however, clearly demonstrate the feasibility of modulating the extent of hyperoxic injury in the alveolar epithelium by supplementing the protective antioxidant mechanisms. The investigators reported that alveolar type II epithelial cells in vitro will take up the liposomes and their contents. They also showed that intratracheal instillation of the liposomes into healthy rats resulted in the distribution of superoxide dismutase and catalase to all lung lobes.

The investigators reported several additional interesting observations during the course of their experiments. They determined that a key enzyme for their cytotoxicity assays, lactate dehydrogenase, was inactivated by hyperoxic and oxidant exposure and, therefore, reduced the detectable levels of this enzyme. As a result, they subsequently altered their assay marker and used radiolabeled adenine. In another series of experiments, the investigators examined the activity of xanthine oxidase and its potential as an intracellular source of reactive oxygen molecules. Results from other studies indicated that oxidant exposures may affect surfactant synthesis by alveolar type II epithelial cells.

In summary, the investigators provided a number of new observations about the feasibility of supplementing the antioxidant capacity of epithelial cells in the lungs with lipo-

somes. They demonstrated that liposomes can effectively deliver antioxidants to epithelial cells both in vitro and in vivo. Because inhalation of air pollutants can exert an oxidant stress on the lungs, the cellular and molecular responses to oxidants in the lungs and mechanisms for protecting lung cells from their damage will continue to be an area for active and innovative research.

REFERENCES

Cullis PR, Hope MJ, Bally MB, Madden TD, Mayer LD, Janoff AS. 1987. Liposomes as pharmaceuticals. In: Liposomes. From Biophysics to Theraputics (Ostro MJ, ed.) Marcel Dekker, New York, NY.

Chang LY, Graham JA, Miller FJ, Ospital JJ, Crapo JD. 1986. Effects of subchronic inhalation of low levels of nitrogen dioxide. Toxicol Appl Pharmacol 83:46–61.

Crapo JD. 1986. Morphologic changes in pulmonary oxygen toxicity. Annu Rev Physiol 48:721–731.

Crapo JD, DeLong DM, Sjöstrom K, Hasler GR, Drew RT. 1977. The failure of aerosolized superoxide dismutase to modify pulmonary oxygen toxicity. Am Rev Respir Dis 115:1027–1033.

Crapo JD, Miller FJ, Mossman B, Pryor WA, Kiley JP. 1992. Environmental lung diseases: Relationship between acute inflammatory responses to air pollutants and chronic lung disease. Am Rev Respir Dis 145:1506–1512.

Dobbs LG, Williams MC, Brandt AE. 1985. Changes in biochemical characteristics and pattern of lectin binding of alveolar type II cells with time in culture. Biochim Biophys Acta 846:155–166.

Dobbs LG, Williams MC, Gonzalez R. 1988. Monoclonal antibodies specific to apical surfaces as rat alveolar type I cells bind to surfaces of cultured, but not freshly isolated, type II cells. Biochim Biophys Acta 970:146–156.

Donato SI, Zabski SM, Crandall ED. 1992. Reactivity of alveolar epithelial cells in primary culture with type I cell monoclonal antibodies. Am J Respir Cell Mol Biol 6:296–306.

Freeman BA, Crapo JD. 1982. Free radicals and tissue injury. Lab Invest 47:412–426.

Gabizon A, Papahadjopoulos D. 1992. The role of surface charge and hydrophilic groups on liposome clearance in vivo. Biochim Biophys Acta 1103:94–100.

Lippmann M. 1989. Health effects of ozone: A critical review. J Air Pollution Control Assoc 39:672–695.

Mason RJ, Dobbs LG. 1980. Synthesis of phosphatidylcholine and phosphatidylglycerol by alveolar type II cells in primary culture. J Biol Chem 255:5101–5107.

Morrow PE. 1984. Toxicological data on NO_x: An overview. J Toxicol Environ Health 13:205–227.

Mustafa MG, Tierney DF. 1978. Biochemical and metabolic changes in the lung with oxygen, ozone, and nitrogen dioxide toxicity. Am Rev Respir Dis 118:1061–1090.

National Research Council. 1992. Rethinking the Ozone Problem in Urban and Regional Air Pollution. National Academy Press, Washington, DC.

Pryor WA. 1991. Can vitamin E protect humans against the pathological effects of ozone in smog? Am J Clin Nutr 53:702–722.

Pryor WA, Church DF. 1991. Aldehydes, hydrogen peroxide and organic radicals as mediators of ozone toxicity. Free Rad Biol Med 11:41–46.

Samet JM, Utell MJ. 1990. The risk of nitrogen dioxide: What have we learned from epidemiological and human studies. Toxicol Ind Health 6:247–262.

Shannon JM, Emrie PA, Fisher JH, Kuroki Y, Jennings SD, Mason RJ. 1990. Effect of a reconstituted basement membrane on expression of surfactant proteins in cultured adult rat alveolar type II cells. Am J Respir Cell Mol Biol 2:183–192.

Shannon JM, Jennings SD, Nielson LD. 1992. Modulation of alveolar type II cell differentiated function in vitro. Am J Physiol 262:427–436.

Turrens JF, Crapo JD, Freeman BA. 1984. Protection against oxygen toxicity by intravenous injection of liposome-entrapped catalase and superoxide dismutase. J Clin Invest 87–95.

U.S. Environmental Protection Agency. 1982. Air Quality Criteria for Nitrogen Oxides. EPA-600/8–82–026F. Environmental Criteria and Assessment Office, Research Triangle Park, NC.

U.S. Environmental Protection Agency. 1986. Air Quality Criteria for Ozone and Other Photochemical Oxidants, Vol. 1 and 2. EPA-600/8–84–02aF and EPA-600/8–84–02bF. Environmental Criteria and Assessment Office, Research Triangle Park, NC.

U.S. Environmental Protection Agency. 1991. National Air Quality and Emissions Trends Report, 1990. Office of Air Quality Planning and Standards, Research Triangle Park, NC.

Weibel ER. 1984. The Pathway for Oxygen: Structure and Function in the Mammalian Respiratory System. Harvard University Press, Cambridge, MA.

Whisett JA, Weaver T, Hull W, Ross G, Dion C. 1985. Synthesis of surfactant-associated glycoprotein A by rat type II epithelial cells. Primary translation products and post-translational modification. Biochim Biophys Acta 828:162–171.

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