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ANNUAL REPORT

**MECHANISMS AND MODIFICATIONS OF PULMONARY AND
SYSTEMIC EPITHELIA FUNCTION AND STRUCTURE BY
REACTIVE OXYGEN SPECIES AND PROTEASES**

N00014-91-J-1277

**Principal Investigator
Dr. Sadis Matalon**

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ANNUAL REPORT**MECHANISMS AND MODIFICATIONS OF PULMONARY AND SYSTEMIC
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AND PROTEASES.**

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Scientific Progress**Overall Purpose**

A number of pathologic conditions including battlefield injuries, skin burns, decreased organ perfusion, sepsis and hemorrhagic shock, result in the release of reactive oxygen species (ROS) in the circulation. In addition, activated neutrophils, aggregating in the site of injury, also release ROS and proteases. Epithelial tissues are prime targets for these species. This injury may lead to electrolyte imbalance, edema, and ultimately death. Our general goal is to identify the mechanisms by which ROS and proteases injure the intestinal and lung epithelia. Active sodium (Na^+) transport is a seminal function of epithelial tissues, and sodium channels are the primary regulated proteins through which Na^+ enters most epithelial cells. Recent evidence from our laboratories indicates the existence of a new type of channel in alveolar epithelial cells. The main goals of the proposed research are to: 1) characterize the biophysical and functional properties of the alveolar Na^+ channels and compare them to those found in intestine and kidney; 2) identify their molecular structure; 3) quantify the effects of intracellular

and extracellular increases in reactive oxygen species and proteases on the function and structure of epithelial and intestinal Na^+ channels; and 4) understand the importance of active Na^+ transport in limiting pulmonary edema in healthy and diseased lungs in vivo. Finally, we will test the hypothesis that an increase in epithelial antioxidant defenses, by the delivery of liposome-encapsulated superoxide dismutase and catalase, mitigate the onset and progression of reactive oxygen species induced injury. The results of these studies will offer new fundamental knowledge on the structure and function of epithelial channels and their role in lung fluid balance. They will also help us devise strategies for pharmacological interventions to limit systemic and epithelial injury and the extent of alveolar edema in following oxygen toxicity and ARDS-type injuries resulting from battlefield wounds.

Military Significance

It has been known that patients with severe battlefield wounds, hemorrhagic shock, extensive skin burns, sepsis and other forms of injury often develop severe lung and intestinal injury. It has been suspected that this injury results from reactive oxygen species and proteases released from the injured site and transported via the blood stream to all parts of the body. In this application, we have proposed a series of studies to quantify the effects of these species on Na^+ transport, a critical function regulating *solute flux* across the pulmonary and intestinal epithelia both in vivo and in vitro.

Signal transduction by neurotransmitters, hormones, growth factors, intracellular ions, and G-proteins involves binding to specific receptors with subsequent translation into a cellular response invariably modulated by alterations in ion channel conductance. Alterations in receptors and channels are probably implicated in the pathophysiology of most disease states. Therefore, receptors and channels are important in modern medicine because they represent the sites of action for many therapeutic agents. Application of molecular genetic techniques to the study of ion channels has provided valuable insights into channel structure-function parameters. In fact, knowledge of channel primary structure and regulation deduced from molecular biological techniques can provide a paradigm for understanding the etiology of ion channel dysfunction in disease as well as in active battlefield injury and other reactive oxygen species induced injury. Molecular biological knowledge of epithelial Na^+ channels will also help devise strategies for pharmacologic and/or immunologic intervention to help curb the extent of injury.

To understand the molecular mechanisms underlying the selective ion transport and maintenance of lung fluid balance by epithelial sodium channels, it is essential to elucidate the primary structure of the constituent protein and to understand the control pathways involved in regulating channel expression. The only technically feasible way to achieve these goals is to use recombinant DNA techniques to clone and sequence DNA complementary to the messenger RNA coding for this Na^+ channel protein. Furthermore, the detailed molecular mechanism of physiological functions can be ascertained by genetic engineering approaches that selectively

modify specific nucleotides in the gene. This allows the production of discrete changes in the amino acid sequence of the encoded protein and, ultimately, correlation of the altered structure to function. Once this is understood, gene transfer experiments can be done to insert functional Na⁺ channel genes into tissues where altered channel activity exists, so as to overcome abnormalities induced by, for example, free oxygen radicals. This project is designed to begin to accomplish these goals. Dr. Benos' laboratory has developed unique biological reagents and processes. This expertise to understand this formidable but extremely essential project. The results will offer new insights as to the nature of the Na⁺ channels, the way they are modified by reactive oxygen species, and will help to establish new rational approaches by which this injury may be ameliorated. For example, circulating concentrations of reactive oxygen species and proteases may be reduced by the intravenous administration of antioxidant enzymes and antiproteases. Alternatively, lung antioxidant tissue content can be enhanced by the intratracheal instillation of liposome encapsulated antioxidant enzymes. Depleted stores of surfactant may be replenished by exogenous surfactant replacement. Compromised active solute transport may be augmented by drugs that increase intracellular stores of cAMP (epinephrine). It is expected that the results of this study will form the rational basis for extensive clinical trials to evaluate the effectiveness of specific agents in the prevention of pulmonary and intestinal injury following wound injury and repair.

Currently, high concentrations of oxygen are used extensively both for the correction of systemic hypoxemia and in decompression tables of the U.S. Navy. However, its usefulness is limited by its well known toxicity to both pulmonary and systemic tissues. Understanding the mechanisms of this injury and limiting its toxicity may allow us to considerably expand the therapeutic uses of this very important agent.

Introduction

During this first year, both projects have made good progress in fulfilling the specific aims of this grant. As can be seen from the attached list, we have published seven papers in peer-reviewed journals, two invited book chapters and seventeen abstracts. In addition, because of the close interactive environment and collaborative studies fostered by this center grant, we have been able to prepare a number of grant applications, three of which have been funded (see pages 17-18). Key scientific accomplishments are summarized in individual projects. In brief, we are making excellent progress in both characterizing and cloning a newly described Na channel, which we demonstrated that it exists in the alveolar epithelium. We also demonstrated that reactive oxygen species damage epithelial cells and administration of PEG-catalase decreases this injury. Finally we conclusively demonstrated that peroxynitrite, a newly described toxic specie released by alveolar macrophages, causes significant injury in colonic enterocytes. These findings may have a significant impact in our understanding of the basic mechanisms leading to

multiple organ failure in ARDS. Based on these findings we have developed clinical protocols to test the efficacy of a number of agents in mitigating the severity and mortality of ARDS in patients.

Project 1

Principal Investigator: Dr. Dale Benos

Summary of Research Activities for 1991 - 1992 (Year 01)

One of the main goals of this project is to elucidate the molecular basis for regulation of amiloride-sensitive Na⁺ channel expression in pulmonary epithelia under conditions of development, hormonal stimulation, and exposure to noxious stimuli. During the past year our research efforts have focused on four major areas: 1) generating cDNA probes for amiloride-sensitive Na⁺ channels in the lung; 2) examining developmental expression of pulmonary Na⁺ and Cl⁻ channel proteins; 3) providing evidence for an amiloride-binding protein in adult pneumocytes; and 4) biochemical characterization of this low amiloride affinity binding protein.

1. Generating cDNA probes for amiloride-sensitive Na⁺ channels in the lung.

There are no nucleotide probes currently available for the amiloride-sensitive Na⁺ channel in pulmonary epithelium. Such probes will be useful for cloning and localizing the channel, and in studying the effects of various perturbations on epithelial Na⁺ transport. As a first attempt at designing such a probe, we have generated a 798 bp oligonucleotide (Habp1) from a human kidney cDNA library with homology to a putative human amiloride-binding protein (Barbry et al., PNAS USA 87:7347-7351, 1990) using sequential, "nested" polymerase chain reactions (PCR). Homology to the published sequence was checked and confirmed with restriction endonuclease digestion and internal oligonucleotide hybridization. Nucleotide sequencing of Habp1 is currently underway to provide definitive confirmation of homology. Habp1 was biotinylated by substitution of biotin-14-dUTP for dTTP in a PCR reaction for use as a probe. The sensitivity of chemiluminescent detection for this probe was established and, currently, hybridization conditions for Northern analysis of rat lung mRNA are being optimized.

A second approach in the identification of a pulmonary epithelial Na⁺ channels uses antibody recognition of a fusion protein from a rat lung cDNA library insert into lambda phage. This work is being done in collaboration with Youngsuk Oh and Randy Baker who used a polyclonal antibody raised against purified bovine renal papillae Na⁺ channel proteins to screen a rat lung cDNA library inserted into a lambda phage. We have isolated plasmid DNA from

positive clones, and we are in the process of analyzing the DNA sequence. Approximately 90% of the plasmid DNA has been sequenced; only two nucleotide segments of about 300 bp have any degree of homology (and only to a DNA binding protein in the coat of lambda bacteriophage). There is a segment of approximately 100 bp at the 5' end of the insert which has no homology to sequences currently in GenBank. The sequence of the remaining middle portion of the insert is yet to be determined. We plan to construct a probe from the 5' region of the insert and perform a) Northern analysis and b) RNA PCR using rat lung mRNA to determine whether the sequence is from the lung. If the sequence is pulmonary in origin, then we will rescreen the rat lung cDNA library to identify a positive clone. However, to be successful, it may be necessary to construct a new, size-fractionated cDNA library.

2. Developmental expression of pulmonary epithelium.

The second major area of research we have pursued is an examination of the effect of developmental maturity on the expression of epithelial ion channels in rat lungs. The abrupt transition from placental to pulmonary gas exchange may correlate with a transition from a Cl⁻-secreting epithelium to a Na⁺ absorbing epithelium. Thus, fetal lung tissue may provide an excellent model for the study of the regulation of epithelial ion transport in the lung. Western analyses of lung homogenate proteins from rats of 17 days gestation through 3 days post birth demonstrated that epitopes recognized by the Na⁺ channel antibody increase with gestational age. Proteins with molecular masses of around 70 kDa, 150 kDa, and >250 kDa appear to demonstrate these developmental changes in preliminary studies. An antibody raised against a bovine tracheal Cl⁻ channel also recognizes epitopes in the lung. The Cl⁻ channel antibody recognized a protein around 60 kDa in the trachea and in lung tissue from 17 through 21 days gestation and postnatal day 1 rat. This protein was also detected in adult lung, although it was faint. Additional proteins around 90 kDa were recognized by the Cl⁻ channel antibody in fetal and early postnatal lung, but not adult tracheal or lung tissue. The significance of these 90 kDa proteins is not known at this time.

In addition to Western analyses, we have performed immunohistochemistry on frozen sections (3-4µm) from fetal (18 days and 22 days gestation) and adult rat lungs and in adult rat trachea. We observed specific staining of the tissues for both antibodies. We are currently optimizing primary and secondary antibody concentrations and conducting a detailed qualitative and quantitative analyses of antibody binding.

3. Providing evidence for an amiloride-binding protein in adult pneumocytes.

Recently low amiloride affinity sodium conductive pathways have been described in freshly isolated rat and rabbit alveolar type II cells. We have found that: 1) polyclonal antibodies raised against high amiloride affinity sodium channel proteins from bovine kidney cross-react with a 135 kDa protein in alveolar type II cell membrane vesicles on Western blots;

2) using the photoactive amiloride analog, NMBA, in combination with anti-amiloride antibodies, we found that NMBA specifically labelled the same 135 kDa protein; and 3) monoclonal anti-idiotypic antibodies directed against polyclonal anti-amiloride antibodies also recognized the same molecular mass protein on Western blots. Immunocytochemical studies using antibodies against high amiloride affinity sodium channels showed that antibody binding was confined to the apical membranes of freshly isolated rat alveolar type II cells. These observations indicate that alveolar type II cell pneumocytes express an amiloride binding protein with an apparent molecular mass of 135 kDa. This protein may be a component of the low amiloride affinity sodium channel that exists in this cell type.

4. Biochemical characterization of this low amiloride affinity binding protein.

This low amiloride affinity binding site in rabbit alveolar type II cells was characterized using [³H]-bromobenzamil as a ligand. [³H]-bromobenzamil (14.4 Ci/mmol) was prepared and used to titrate binding sites in both bovine kidney papillary membranes and adult rabbit alveolar type II cell membranes. Scatchard analyses of binding of the [³H]-bromobenzamil binding to the membranes of bovine kidney and alveolar type II cells showed a single affinity binding site with a K_d of 50 nM and 370 nM, respectively. The maximum number of binding sites in the alveolar type II cells was 12.5 pmol/mg of protein. In pneumocytes, EIPA displaced the bound probe more effectively than amiloride or benzamil (apparent K_i = 5, 40, 100 μM for EIPA, amiloride, and benzamil, respectively), in agreement with previous ²²Na⁺ uptake studies in alveolar type II cell membrane vesicles (AJP 260:L90, 1991). It is known that the structure/inhibitory pattern for high affinity amiloride binding sites is benzamil > amiloride >> EIPA, opposite of what we observed. In addition, we have photolabelled the benzamil binding site rat alveolar type II cells with the photoreactive amiloride analog NMBA, and found that NMBA specifically photoincorporated into a polypeptide with an apparent molecular mass of 135 kDa. The photoincorporation of NMBA into this peptide is competitively inhibited by high concentrations of unlabelled benzamil. Thus, these cells express a low amiloride affinity benzamil binding site with an apparent molecular mass of 135 kDa.

Research Plan For the Next Granting Period

In addition to continuing work along the four projects listed above, we plan to examine the role of defective cAMP-mediated surfactant secretion in CF related pulmonary pathophysiology, specifically using molecular techniques to determine whether CFTR is present in alveolar type II cells. We will then compare constitutive and related surfactant secretion in CFTR-competent and defective lungs or isolated cells. Lungs will be made CFTR-defective by anti-sense oligonucleotide transfection.

We will further characterize the low amiloride affinity binding protein that we have isolated. Our plan is to isolate large quantities of this protein, and do amino acid sequence analysis by standard techniques to get an idea of the primary amino acid structure of the amiloride/benzamil binding site. We have also prepared some synthetic peptides against consensus sequences of all known amiloride binding proteins. Antibodies will be generated against this peptide for identification and immunoaffinity purification in lung cells. The protein purified by standard biochemical techniques, as well as by immunoaffinity chromatography, will be incorporated into planar lipid bilayers and structure/function activities monitored in single channel studies.

Project 2

Principal Investigator: Dr. Sadis Matalon

Progress Report (March 1991-February 1992).

The general objectives of this project are: (1) to identify sensitive indices that will allow the quantification of injury to the pulmonary and colonic epithelial by ROS, peroxynitrite and proteases; (2) to determine the physiological consequences of this injury on lung fluid balance; (3) elucidate the mechanisms that may explain the observed effects and (4) devise pharmacological interventions to limit the extent of injury to these tissues.

During this last year we have made excellent progress in setting up experimental models and designing experiments that will allow us to fulfill the specific aims outlined in the original proposal. Key experimental findings are summarized below and described below:

Summary of Experimental Findings

1. We have demonstrated the existence of a new family of sodium channels in alveolar epithelial cells. This discovery may allow us to gain significant new insight as to the mechanisms regulating fluid and electrolyte transport across the normal and injured epithelium.

2. We have identified key biochemical mechanisms that explain how free radicals interfere with surfactant metabolism in alveolar type II pneumocytes and demonstrated, for the first time, that increasing ATII cell catalase content, by incubating these cells with polyethylene glycol conjugated catalase, prevents this injury. This observation has important implications in the prevention and treatment of Adult Respiratory Distress System injury in humans.

3. We were one of the first groups to demonstrate the physiological significance of peroxynitrite, a newly described reactive oxygen specie, by demonstrating that it decreases active sodium transport across both apical membrane vesicles of colonic cells and freshly isolated ATII cells. This observation may have important implications in our understanding of the basic mechanisms of free radical injury in tissues and devising ways to ameliorate it.

4. We have demonstrated the development of "distal" lung injury following clamping and unclamping of the descending aorta. This commonly used surgical procedure results in ischemia-reperfusion of large organs and tissue beds and validates our initial hypothesis that systemic injury may result in ARDS. We also demonstrated that reactive oxygen species generated by xanthine and xanthine oxidase, cause increased permeability of the pulmonary vasculature.

Detailed Description of Experimental Findings.

1. Identification and Functional Characterization of Na⁺ conductive pathways in adult and fetal alveolar type II (ATII) pneumocytes.

The purpose of this study was to document the existence, assess the spatial localization, and characterize some of the transport properties of proteins antigenically related to epithelial Na⁺ channels, in freshly isolated rabbit and rat alveolar type II (ATII) cells. ATII cells, isolated by elastase digestion of lung tissue and purified by density-gradient centrifugation, were incubated with polyclonal antibodies raised against Na⁺ channel protein purified from beef kidney papilla (NaAb), followed by a secondary antibody (goat antirabbit IgG conjugated to FITC). Rat ATII cells exhibited specific staining with NaAb at the level of the plasma membrane (Fig. 1), which, in most cells, colocalized with that of the lectin Maclura Pomiferra Agglutinin, an apical surface marker. In Western blots, NaAb specifically recognized a 135 ± 10 kDa protein in rat ATII membrane vesicles. When patch-clamped in the whole-cell mode using symmetrical solutions (150 mM Na⁺-glutamate), ATII cells exhibited outwardly-rectified Na⁺ currents which were diminished by amiloride (10-100 μM), instilled into the bath solution. Ion substitution studies showed that the conductive pathways were three times more permeable to Na⁺ than K⁺. Amiloride, benzamil and EIPA were equally effective in diminishing ²²Na⁺ flux into rabbit and rat ATII cells (45% inhibition at 100 μM, with IC50 of about 1 μM for all inhibitors). TEA (10 mM) or BaCl₂ (2 mM), well-known K⁺- channel blockers, had no effect on ²²Na⁺ uptake. These results indicate that ATII cells express an amiloride-sensitive Na⁺ conductance, probably a channel, with a lower affinity for amiloride and its structural analogues, than the well established amiloride sensitive Na⁺ channels found in bovine renal papilla and cultured amphibian A6 kidney cells. *Since Na⁺ channels conduct at rates far exceeding that of any other Na⁺ transporter, they may be one of the most important pathways for the entry of Na⁺ into alveolar epithelial cells during its active transport across the blood-gas barrier. Presently, we are conducting studies to assess the hormonal regulation of these conductive*

pathways. The results of these studies may have important implications in limiting the degree of pulmonary edema in Adult Respiratory Distress Syndrome.

In our next study, we sought to characterize the pathways through which Na^+ enters the apical membrane of fetal alveolar pneumocytes (FATII). The reason we did this study was to attempt to understand the basic mechanisms of fetal alveolar fluid that takes place shortly after birth. FATII were isolated from the lungs of 20 day gestation fetal rats as previously described, plated on T-75 flasks and maintained in 95/5% air/ CO_2 37°C for 48 h. They were then scraped from the flasks and an enriched plasma membrane vesicle fraction (MV) was obtained by differential centrifugation. Carrier-free $^{22}\text{Na}^+$ uptake into membrane vesicles from these cells was measured in the presence of an inside negative membrane potential, produced by the addition of the K^+ ionophore valinomycin (val; 10 μM) after all external K^+ was removed by cation exchange chromatography. Electrogenic $^{22}\text{Na}^+$ uptake (ELNa) was defined as the difference in intravesicular radioactivity in the presence and absence of val. FATII MV exhibited considerably higher levels of ELNa as compared to the corresponding value for adult ATII rat MV (72 ± 14.5 vs 14.4 ± 0.41 ; % of added radioactivity/mg protein; values obtained 5 min after the addition of $^{22}\text{Na}^+$). Amiloride and two of its structural analogues, EIPA and benzamil, were equally effective in reducing FATII MV ELNa values with K_i 's of about 10 μM . It is concluded that a fraction of Na^+ transport across fetal ATII cells occurs through conductive pathways with low affinity to amiloride. These pathways have similar functional characteristics with those described in adult rat ATII cells.

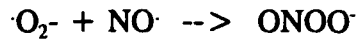
2. Mechanisms of Free Radical Injury to epithelial cells and tissues.

a. Free Radical Inhibition of Active Na^+ Transport across Colonic Enterocytes.

Increased concentrations of reactive oxygen species compromise both active (transcellular) and passive (paracellular) solute transport across a variety of epithelial tissues. For example, it has been demonstrated that exposure to hyperoxia or xanthine and xanthine oxidase (XO) decreases the short circuit current, an index of sodium (Na^+) transport, across the toad bladder and ventral toad skin respectively. However, these studies did not address the underlying mechanisms. Neither did they identify which free radical species were responsible for this injury. Since the entry of Na^+ across the apical membrane of epithelial cells is the rate limiting step in transcellular Na^+ transport, and amiloride-blockable Na^+ channels are the principal proteins regulating this process in most epithelial cells, we hypothesize that injury to these proteins by reactive oxygen species may contribute to the observed decrease in Na^+ transport.

Recently, a second pathway for the generation of strong oxidants with the reactivity of $\cdot\text{OH}$ has been described. The entry of calcium into endothelium and neurons triggers the synthesis and release of the stable free radical nitric oxide ($\text{NO}\cdot$) as a secondary

messenger which mediates vasodilation and reduces platelet adhesion. Stimulated macrophages and neutrophils also produce both $\cdot\text{O}_2^-$ and NO . Because $\cdot\text{O}_2^-$ and NO contain unpaired electrons, they rapidly react to form peroxynitrite (ONOO^-), a reactive and potentially toxic species:



Peroxynitrite has a half-life of under 1 sec at pH 7.4 at 37°C, because peroxynitrous acid (ONOOH) spontaneously decomposes to give $\cdot\text{OH}$ and nitrogen dioxide (NO_2):



In spite of its reactivity, the modest rate of decomposition under physiological conditions allows ONOO^- to diffuse for up to several cell diameters. Peroxynitrite and its by-products initiate lipid peroxidation and oxidize sulfhydryl groups, and therefore may contribute significantly to partially reduced oxygen species injury.

Peroxynitrite (ONOO^-) is a potent oxidizing agent that initiates lipid peroxidation and sulfhydryl oxidation and may be responsible for a portion of the cytotoxicity attributed to superoxide anion (O_2^-). We quantified the extent to which ONOO^- , xanthine plus xanthine oxidase (XO) and hydrogen peroxide (H_2O_2), decreased sodium (Na^+) uptake into membrane vesicles derived from colonic cells of dexamethasone-treated rats. Previous studies have shown that 80 - 83% of Na^+ uptake into these vesicles occurs through amiloride-blockable Na^+ channels. Carrier free $^{22}\text{Na}^+$ uptake into vesicles was measured in the presence of an inside-negative membrane potential, produced by the addition of the potassium ionophore valinomycin (10 μM) after removal of all external potassium by cation exchange chromatography. Preincubation of vesicles with either 100 μM or 1 mM ONOO^- for 30 s decreased the amiloride-blockable fraction of Na^+ uptake by $27 \pm 7\%$ and $65 \pm 2\%$ respectively ($X \pm \text{SEM}$; $n \geq 5$; $p < 0.05$ from control). However, the amiloride-insensitive part of Na^+ uptake was not affected, indicating that there was no overt destruction of these vesicles by these ONOO^- concentrations. Decomposed ONOO^- hydrogen peroxide (1 μM - 10 mM), or xanthine (500 μM) plus XO (10 - 30 mU/ml), either in the absence or in the presence of 100 μM FeEDTA, did not decrease Na^+ uptake.

These data indicate that ONOO^- is a potent injurious agent that can compromise Na^+ uptake across epithelial cells, possibly by damaging Na^+ channels. They also clearly demonstrate that in our system that even supraphysiological concentrations of superoxide, hydrogen peroxide and hydroxyl radicals did not affect the values of these variables. They thus have forced us to reevaluate our basic understanding of free radical tissue injury and of ways to prevent it.

b. Mechanisms and modification of H_2O_2 injury to the surfactant system of ATII cells.

Alterations in type II pneumocyte function, including surfactant biosynthesis, may play a significant role in the development and pathophysiology of oxidant-induced lung injury. The results of this study showed that exposure of type II cells to amounts of H_2O_2 released by activated neutrophils and other inflammatory cells, caused a dose dependent decrease in PC synthesis with only minimal changes in cell viability. In addition, the specific activities of the cholinephosphate cytidyltransferase and cholinephosphotransferase, specific enzymes of phosphatidylcholine synthesis, were not significantly decreased by the oxidant exposure. However, the activity of glycerolphosphate acyltransferase, a sulfhydryl dependent enzyme involved in an early stage of phospholipid synthesis, was decreased by hydrogen peroxide in a dose-dependent manner that was similar to that seen for PC synthesis. Further studies showed that incubation of type II cells with polyethylene glycol (PEG)-conjugated catalase for one hour resulted in an increased in the cellular catalase content (53 ± 5 units/mg protein vs. 6.7 ± 1.5 units/mg protein for controls). *More importantly, these cells showed no change in PC synthesis rates when incubated with $300 \mu M H_2O_2$* (0.568 ± 0.06 nmol/106 cells/hr vs. 0.505 ± 0.03 vs. 0.505 ± 0.03 for controls). These results indicated that: 1) oxidant mediated decreased in surfactant biosynthesis may be due to specific inhibition of key enzymes of phospholipid metabolism; and 2) the deleterious effects of oxidant stress on type II cell surfactant synthesis may be pharmacologically modified in vitro, a concept that may have utility with regard to the modulation of in vivo induced oxidant lung injury.

c. Peroxynitrite Inhibition of Oxygen Consumption and Ion Transport in Alveolar Type II (ATII) Pneumocytes.

As mentioned above, it has been recently demonstrated that activated alveolar macrophages secrete both nitric oxide (NO) and superoxide (O_2^-) in the alveolar lining fluid. These agents contain unpaired electrons and combine rapidly to form peroxynitrite (ONOO $^-$), a potent oxidizing agent causing lipid peroxidation and sulfhydryl oxidation in biological membranes. In this study we quantified ONOO $^-$ cytotoxicity in freshly isolated ATII cells by measuring: (1) KCN dependent and independent oxygen consumption (VO_2); (2) amiloride ($10 \mu M$) inhibitable $^{22}Na^+$ uptake (Am- $^{22}Na^+$), as an index of Na^+ entry through low-amiloride affinity Na^+ channels; and (3) ouabain ($100 \mu M$)-sensitive $^{86}Rb^+$ uptake (Ou.- $^{86}Rb^+$), as an index of Na^+ - K^+ -ATPase activity in these cells. ATII cells were isolated to purity of $>85\%$ by elastase digestion of lung tissue, followed by Percoll centrifugation. Cell associated $^{22}Na^+$ and

$^{86}\text{Rb}^+$ radioactivity was quantified by incubating ATII cells with trace amounts of $^{22}\text{NaCl}$ and $^{86}\text{RbCl}$ for 10 min, and removing extracellular radioactivity by pelleting cells through a phthalate cushion. Mean values of these variables (expressed as % of control) are shown below:

ONOO $^-$ μM	0	50	100	250	500
VO $_2$ -KCN	100	94	79	58	33
VO $_2$ +KCN	100	89	92	70	11
Am- $^{22}\text{Na}^+$	100	91	90	--	47
Ou. $^{86}\text{Rb}^+$	100	114	106	--	111

Exposure to ONOO $^-$ did not alter ATII cell viability. It was concluded that ONOO $^-$ inhibits ATII cell respiration, decreases Na^+ entry into ATII cells by damaging apically located Na^+ channels, but does not inhibit Na^+ - K^+ -ATPase activity.

d. Mechanisms of Extracellular Reactive Oxygen Species Injury to the Pulmonary Microvasculature.

We investigated the effect of xanthine (X) plus xanthine oxidase (XO) on pulmonary microvascular endothelial permeability in isolated rabbit lungs perfused with Krebs buffer containing bovine serum albumin (5 wt%). Addition of five mU/ml XO and 500 μM xanthine to the perfusate caused a two-fold increase in the pulmonary capillary filtration coefficient (Kf,c), 30 min later without increasing the pulmonary capillary pressure. This increase was prevented by allopurinol or catalase, but not by superoxide dismutase, or dimethylsulfoxide. Since this data implicated hydrogen peroxide (H_2O_2) as the injurious agent, we measured its concentration in the perfusate after the addition of X and XO for a 60 min interval. In the absence of lung tissue and albumin, H_2O_2 increased with time reaching a concentration of about 250 μM by 60 min. If albumin (5 wt%) was added to the perfusate, or in the presence of lung tissue, the corresponding values were 100 μM and less than 10 μM respectively. To understand the mechanisms of H_2O_2 scavenging by lung tissue we added a 250 μM bolus of H_2O_2 in the lung perfusate. We found that H_2O_2 was removed rapidly with a half-life of 0.31 ± 0.04 min ($X \pm 1$ SE). This variable was not increased significantly by inhibition of lung catalase activity with sodium azide or inhibition of the lung glutathione redox cycle with 1-chloro-2,4-dinitrobenzene. However, inhibition of both enzymatic systems

increased the half-life of H_2O_2 removal to 0.71 ± 0.09 min ($X \pm 1$ SE; $p < 0.05$). These studies indicate that the lungs can remove large quantities of H_2O_2 by both enzymatic and non-enzymatic processes, but that this scavenging does not prevent the induced injury to the pulmonary microvasculature.

e. Lung Injury after clamping and unclamping of the aorta.

A clinically relevant and unique model of closed chest aortic crossclamping (CCAC) was designed to examine lung injury after systemic ischemia-reperfusion. Anesthetized rabbits with controlled ventilation via tracheostomy were invasively monitored to document hemodynamic events during CCAC. CCAC was performed by placement of a 4 f Fogarty catheter via the femoral artery into the thoracic aorta, just above the diaphragm, and expanding the balloon. After 30 min of CCAC, during 2 h of reperfusion, declamping shock was treated with sodium bicarbonate and phenylephrine (BP group) to maintain acid-base and hemodynamic status at pre-CCAC levels. There were no differences between sham or BP groups' peak inspiratory pressures, alveolar-arterial oxygen tension gradients, or lung wet to dry weight ratios. Central venous/pulmonary arterial pressures in the BP group did not increase during CCAC. In contrast, bronchoalveolar lavage albumin concentrations (mg/kg/lung) were significantly greater in the BP group ($n=5$) compared to sham ($n=5$) animals ($7.23 \pm .35$ vs 2.72 ± 1.76 ; $x \pm 1$ SEM, $p < 0.05$). These data suggest that CCAC followed by reperfusion increases alveolar capillary membrane permeability without significantly changing clinical parameters of lung function. Intravenous allopurinol, an inhibitor of xanthine oxidase, significantly decreased the level of alveolar albumin, indicating the possible involvement of this radical generator, in distal lung injury.

Research Plan for Next Year

There is no major shift in the direction of the proposed studies. We plan to fulfill the specific aims of project 2, as outlined in page 70 of the original application. A list of proposed studies is shown below:

1. We will investigate whether peroxynitrite and other free radicals damage pulmonary surfactant. This will be accomplished by exposing isolated surfactant to various concentrations of peroxynitrite, xanthine and xanthine oxidase in the presence and absence of FeEDTA and measure minimum surface tension in an oscillating bubble. Preliminary results indicate that peroxynitrite prevents surfactant from reaching a minimum surface tension. We will then attempt to identify the cellular and molecular mechanisms responsible for this action. We will also assess whether peroxynitrite-treated surfactant can restore normal lung mechanics in surfactant-deficient lungs.

2. We will quantify the effects of proteases on active Na⁺ transport across both colonic enterocytes and alveolar type II pneumocytes and identify possible synergisms between the actions of proteases and reactive oxygen species.
3. We will quantify the distribution of aerosolized and instilled surfactant and liposome encapsulated antioxidant enzymes in normal and injured lungs. The goal of these studies is to devise the most optimum vehicle for delivering antioxidant enzymes in pulmonary target cells.

Publications

1. Matalon S, Bridges RJ, and Benos DJ. Amiloride-inhibitable Na⁺ conductive pathways in alveolar type II pneumocytes. *Am J Physiol (Lung Cell. & Mol Physiol 4):L90-L96*, 1991.
2. Royall JA, Matalon S. Pulmonary Edema in ARDS. Furmen BT, Zimmerman, JJ, Eds. *Pediatric Critical Care*, St. Louis, MO, C.V. Mosby (In press, 1991).
3. Matalon, S. Mechanisms and Regulation of Ion Transport in Adult Mammalian Alveolar Type II Pneumocytes. *Am J Physiol 261 (Cell Physiol 30):C727-C738*, 1991.
4. Holm BA, Keicher L, Hudak BB, Cavanaugh C, Baker RR, and Matalon S. Mechanisms of H₂O₂-mediated injury to type II pneumocyte surfactant metabolism and protection with PEG-catalase. *Am J Physiol 261 (Cell Physiol 30):C751-C757*, 1991.
5. Bauer ML, Beckman JS, Bridges RJ, Fuller CM, and Matalon S. Peroxynitrite inhibits sodium uptake in rat colonic membrane vesicles. *Biochim Biophys Acta*, in press, 1991.
6. Barnard, ML, Matalon S. Mechanisms of extracellular reactive oxygen species injury to the pulmonary microvasculature. *J Applied Physiol*, accepted, 1991.
7. Matalon S, Kirk KL, Bubien J, Oh Y, Hu P, Yue G, Shoemaker RL, Cragoe EF, and Benos DJ. Immunocytochemical and patch clamp characterization of Na⁺ conductance in alveolar pneumocytes. *Am J Physiol (Cell Physiol)*, in press, 1992.
8. Matalon S, Oh Y, and Benos DJ. Epithelial cell Na⁺ and Cl⁻ channels. *Signal Transduction in Lung Cells*, in press, 1992.
9. Benos, DF, Cunningham SA, Baker RR, Beason KB, Oh Y, and Smith PR. Molecular characteristics of amiloride-sensitive sodium channels. In: *Reviews of Physiology, Biochemistry, and Pharmacology*, in press, 1992.

Abstracts

1. Matalon S, Benos DJ, O'Brodovich H. Amiloride sensitive Na⁺ conductance in membrane vesicles of fetal alveolar type II pneumocytes. *Am Rev Respir Dis* 143:A303, 1991.
2. Hu P, Oh Y, Jilling T, Benos DJ and Matalon S. Immunofluorescent localization of sodium conductance in cultured rat alveolar type II pneumocytes (ATII). *Am Rev Respir Dis* 143:A208, 1991.
3. Baker RR, Jilling T, Matalon S, and Kirk KL. Nonuniform alveolar uptake of liposome-encapsulated CuZn superoxide dismutase and catalase. *Am Rev Respir Dis* 143:A303, 1991.
4. Holm BA, Keicher L, Hudak BB, Baker RR, and Matalon S. Mechanisms of H₂O₂-mediated injury to type II pneumocyte surfactant metabolism and protection with PEG-Catalase. *Am Rev Respir Dis* 143:A741, 1991.
5. Easterling L, Matalon S. Do alveolar macrophages diminish oxidant injury to the alveolar epithelium? Fourth Panamerican and Iberic Congress on Intensive and Critical Care Medicine, May 5-10, 1991, Rio de Janeiro, Brazil.
6. Oh Y, Hu P, Kleyman TR, Saccomani G, Matalon S, and Benos DJ. Evidence for the presence of an amiloride binding protein in adult alveolar type II (ATII) pneumocytes. *FASEB J* 5(4):A690, 1991.
7. Bauer ML, Bridges RJ, Beckman JS and Matalon S. Apical membrane sodium (Na⁺) channels are inhibited by peroxynitrite (ONOO⁺), but not by oxygen radicals. *FASEB J* 5(4):A888, 1991.
8. Nielsen V, Koves T, Matalon S and Gelman S. Lung injury after ischemia-reperfusion by thoracic aorta crossclamping. *FASEB J* 5(5):A1270, 1991.
9. Easterling L, Nielsen V, and Matalon S. A contrast of lung injury after transtracheal instillation of normal saline and buffered solutions. *FASEB J* 5(5):A1397, 1991.
10. Royall JA and Matalon S. Macrophages protect cultured endothelial cells from hydrogen peroxide mediated injury. Pediatric Critical Care Colloquium, January 26-30, 1992, Snowbird, Utah.

11. Nielson V, Matalon S, Holm BA, and Gelman S. Pulmonary injury after ischemia-reperfusion by crossclamping of the thoracic aorta in rabbits. 1992 IARS Meetings, accepted.
12. Hu P, Zhu L, Ischiropoulos H, and Matalon S. Peroxynitrite inhibition of oxygen consumption and ion transport alveolar type II (ATII) pneumocytes. *Am. Rev. Resp. Dis.*, in press, 1992.
13. Hu P, Yue G, Benos D, Shoemaker R, and Matalon S. Characterization of Na⁺ conductive pathways in alveolar type II (ATII) cells. *Am. Rev. Resp. Dis.*, in press, 1992.
14. Yue G, Shoemaker RL, Benos DJ, and Matalon S. Patch clamp characterization of Na⁺ currents (I_{Na}) on cultured rat alveolar type II (ATII) cells. *Am. Rev. Resp. Dis.*, in press, 1992.
15. Royall JA and Matalon S. Macrophages protect cultured endothelial cells from hydrogen peroxide mediated injury. *Faseb J*, in press, 1992.
16. Czopf L, Myles CT, and Matalon S. Fluorescent labelling does not affect the minimum surface tension of surfactant mixtures. *FASEB J*, in press, 1992.
17. Oh Y, Matalon S, Kleyman TR, and Benos DJ. Biochemical characterization of a low affinity benzamil binding site in adult rabbit alveolar type II (ATII) pneumocytes. *FASEB J.*, in press, 1992.

NOTE: As shown by the large number of abstracts, a number of projects are currently in progress. We are confident that a number of full-length papers will be submitted for publication in peer-reviewed journals during the next year.

Grants Funded from Extramural Sources

1. **Baker, Randall R., Ph.D.**
American Lung Association
Annual Direct Costs: \$19,000
Project Period: 07/01/91 - 06/30/93
P.I.: Baker, Randall R., Ph.D.

2. **Matalon, Sadis, Ph.D.**
Burroughs Wellcome Company
Annual Direct Costs: \$35,300
Project Period: 12/01/90 - 11/30/92
P.I.: Matalon, Sadis, Ph.D.

American Lung Association
Annual Direct Costs: \$35,000
Project Period: 09/01/87 - 06/30/92
P.I.: Matalon, Sadis, Ph.D.

NIH #5R01 HL31197-08
Annual Direct Costs: \$116,736
Project Period: 08/01/87 - 07/31/92
P.I.: Matalon, Sadis, Ph.D.

Pending:

NIH #5R01 HL31197-09
Annual Direct Costs: \$150,000
Project Period: 08/01/92 - 07/31/97
P.I.: Matalon, Sadis, Ph.D.
Co-Investigators: Benos, Dale J., Ph.D., and Beckman, J., Ph.D.
(This grant received a percentile score of 7.9% and is expected to be funded at the requested budget level.)

3. **Benos, Dale J., Ph.D.**

NIH-NIDDK #5R01 DK37206-6

Annual Direct Costs: \$105,928

Project Period: 09/01/85 - 06/30/92

P.I.: Benos, Dale J., Ph.D.

(Competitive renewal of this project is pending)

NIH (CF Center Grant Application #1 P50 DK42017-02

Annual Direct Costs: \$397,931

Project Period: 07/01/89 - 06/30/94

P.I.: Benos, Dale J., Ph.D.

NIH-NIDDKD #1T32 DK07545-04 (Training Grant)

Annual Direct Costs: \$98,820

Project Period: 07/01/87 - 06/30/92

P.I.: Benos, Dale J., Ph.D., Co-Investigator: Matalon, Sadis, Ph.D.

(Competitive renewal of this project is pending)

NIH-NIDDK #5P01 DK38518-05 (Project 3)

Annual Direct Costs: \$10,126

Project Period: 04/01/87 - 03/31/92

P.I. Project 3: Benos, D.J., Ph.D.

4. **Beckman, Joseph S., Ph.D.**

NIH R01 HL46407

Annual Direct Costs: \$104,000

Project Period: 04/01/91 - 03/31/94

P.I.: Beckman, Joseph S., Ph.D.

Beckman, Joseph S., Ph.D.

American Heart Association Established Investigator Award

Annual Direct Costs: \$35,000

Project Period: 07/01/90 - 06/30/95

P.I.: Beckman, Joseph S., Ph.D.

Beckman, Joseph S., Ph.D.

American Heart Association

Annual Direct Costs: \$35,000

Project Period: 07/01/90 - 06/30/93

P.I.: Beckman, Joseph S., Ph.D.

DETAILED BUDGET FOR 12-MONTH PERIOD							
DIRECT COSTS ONLY (PROJECT 1)							
Personnel	Role	Type Appt.	% of Appt.	% Effort	Salary	Fringe	Total
Dale J. Benos, Ph.D.	P.I.	1	8	8	\$8,939	\$2,324	\$11,263
Sonia Cunningham, Ph.D.	Coinvest.	1	80	80	\$26,250	\$6,825	\$33,075
Sadis Matalon, Ph.D.	Coinvest.	1	10	10	\$0	\$0	\$0
R. Randall Baker	Res. Assoc.	1	100	100	\$7,250	\$1,885	\$9,135
Peter Smith	Res. Assoc.	1	100	100	\$28,860	\$7,504	\$36,364
Pia Arrote	Sr. Res. Tech.	1	100	100	\$16,800	\$4,368	\$21,168
YoungSuk Oh	Grad. Stud.	1	100	100	\$12,501	\$0	\$12,501
	Subtotal				\$100,600	\$22,906	\$123,506
Consultant Costs							
	Subtotal						\$0
Equipment							
Miscellaneous upkeep for electronic equipment							\$1,000
	Subtotal						\$1,000
Supplies							
1. Rabbits (10 at \$30 per rabbit)							\$300
Daily charges for Upkeep							\$2,000
2. Rats (15 at \$15 per rat)							\$225
Daily charges for Upkeep							\$1,000
3. Isotopes							\$1,000
4. Chemicals							\$1,000
5. Molecular Biological Reagents							\$5,000
6. Tissue Culture Supplies							\$3,000
7. Gel Electrophoresis/Western Blot/ Autoradiography Supplies							\$2,000
8. Scintillation Vials, Glassware, Miscellaneous							\$275
	Subtotal						\$15,800
Travel							
One trip per year for two investigators to a national meeting							\$1,600
	Subtotal						\$1,600
Other Expenses							
Service Contracts for HPLC							\$4,000
	Subtotal						\$4,000
Total Direct Costs for the second year							\$145,906
Indirect Costs for the second year							\$61,280
Total Costs for the second year							\$207,186

Personnel	DIRECT COSTS ONLY (PROJECT 2)			% Effort	Salary	Fringe	Total
	Role	Type Appt.	% of Appt.				
Sadis Matalon, Ph.D.	P.I.	1	25	25	\$26,250	\$6,825	\$33,075
Joseph Beckman, Ph.D.	Coinvest.	1	10	10	\$6,300	\$1,638	\$7,938
Robert J. Bridges	Coinvest.	1	10	10	\$4,200	\$1,092	\$5,292
Dale J. Benos	Coinvest.	1	10	10	\$0	\$0	\$0
Laszlo Czopf	Postdoc	1	50	50	\$10,500	\$2,730	\$13,230
Joyce Haskell	Postdoc	1	100	100	\$30,000	\$7,800	\$37,800
Tanta Myles	Res. Assist.	1	100	100	\$18,800	\$4,888	\$23,688
	Subtotal				\$96,050	\$24,973	\$121,023
Consultant Costs							
None							\$0
	Subtotal						\$0
Equipment							
None							\$0
	Subtotal						\$0
Supplies							
1. Rabbits (200 at \$30 per rabbit)							\$6,000
Daily charges for upkeep							\$2,100
2. Liquid oxygen (30 cylinders at \$100 per cylinder)							\$3,675
3. Chemicals for ATII Isolation							\$4,200
4. Radioactive materials							\$5,250
5. General Chemical Reagents							\$3,150
6. Drugs and surgical supplies							\$3,150
	Subtotal						\$27,525
Travel							
One trip per year for three investigators to a national meeting							\$4,720
	Subtotal						\$4,720
Other Expenses							
Service Contracts for one Gilford Spectrophotometer							\$3,675
Miscellaneous Upkeep for Electronic Equipment							\$525
	Subtotal						\$4,200
Total Direct Costs for the second year							\$157,468
Total Indirect Costs for the second year							\$66,137
Total Costs for the second year							\$223,605

BUDGET JUSTIFICATION: Project 1

Personnel: The personnel for the upcoming budget year are essentially the same as in the first year, except for the addition of another research associate, Peter Smith. Dr. Smith has been in this laboratory for two years, and is quite adept at immuno- and electron-localization techniques. His input in the proposed work of this budget period is essential to accomplish the goals. He will be dedicating 100% of his efforts to this project. Also, the amount of allocated funds for Pia Arrate's salary has been increased. Dr. Randy Baker's salary, but not % effort, has also decreased. Dr. Baker was the successful recipient of an American Lung Association Postdoctoral Fellowship Grant for his work in this proposal and, thus, his salary for the most part is paid for by funds from that award.

Youngsuk Oh, a graduate student, has been purifying and reconstituting low affinity sodium channels from the lung into planar lipid bilayers. Although he was listed on the grant at 100% effort with no salary, his support from the Department of Physiology and Biophysics has been terminated. Thus, his salary needs to be picked up by this grant. He will continue to contribute 100% of his efforts to this proposal.

Sonia Cunningham's salary has been increased 5% to account for cost-of- living increases.

Equipment: \$1,000 is requested for upkeep of electronic equipment and for replacement (plates, power supply elements, etc.) parts for the polyacrylamide and agarose gel electrophoresis systems.

Supplies: The Supply category, initially awarded at \$42,347, has been reduced to \$15,800 to allow for the addition of Dr. Smith to this grant. Of course, this decrease in supply funds will hopefully not seriously compromise the work in progress. Funds will be secured from other sources (mainly departmental) to make up for this shortfall.

Travel and Other Expenses: Both of these categories likewise had to be reduced in order to accommodate the additional funds required for Dr. Smith's, Pia Arrate's, and Youngsuk Oh's salaries. Nonetheless, we feel that we can accomplish most of the goals set out for this upcoming budget year, in spite of the monetary limitations.

BUDGET JUSTIFICATION Project 2

There are no substantial changes from the previously submitted budget. Dr. Joyce Haskell replaced Dr. Robert Garner and Dr. Laszlo Czopf replaced Timothy Koves. Also note because of the increasing personnel costs, the Department of Anesthesiology has agreed to cover the salary of the Administrative Assistant at no additional cost to the Navy.

Figure 1.

Isolated rat ATII pneumocytes exhibit specific immunofluorescent staining with the polyclonal antibody to Na⁺ channel protein (NaAb). Results of a typical experiment.

- a. Fluorescence image of a field of isolated rat ATII pneumocytes incubated with nonspecific rabbit IgG instead of NaAb. Nonspecific immunofluorescent staining was negligible.
- b. DICM image corresponding to (A) indicating the presence of intact cells.
- c. Fluorescence image of isolated rat ATII pneumocytes, incubated with N a A b , illustrating considerable immunofluorescent staining compared to control cells (compare to panel A).
- d. DICM image corresponding to C.

Note: Due to the high cost of photographic services, the figure is submitted in only one copy of the original. Photocopies are provided with all other copies of this document.

