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UMI
MECHANISMS BY WHICH ENDOTOXIN-STIMULATED ALVEOLAR
MACROPHAGES IMPAIR LUNG EPITHELIAL SODIUM TRANSPORT

by

A. John Dickie, M.D.

A thesis submitted in conformity with the requirements for the Degree of Master of
Science in the University of Toronto

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Hospital for Sick Children,
and
Division of General Surgery, Department of Surgery,
Toronto Hospital (General Division),
and
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Toronto, Canada
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<td>A</td>
<td>Amps</td>
</tr>
<tr>
<td>Å</td>
<td>Angstroms</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ALI</td>
<td>acute lung injury</td>
</tr>
<tr>
<td>AM</td>
<td>alveolar macrophage</td>
</tr>
<tr>
<td>ANOVA</td>
<td>one way analysis of variance</td>
</tr>
<tr>
<td>ANP</td>
<td>atrial natriuretic peptide</td>
</tr>
<tr>
<td>ARDS</td>
<td>acute respiratory distress syndrome</td>
</tr>
<tr>
<td>ATI</td>
<td>alveolar type I epithelial cell</td>
</tr>
<tr>
<td>ATII</td>
<td>alveolar type II epithelial cell</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>BSO</td>
<td>buthionine sulfoxamine</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCT</td>
<td>cortical collecting tubule</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>chloride</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>-------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>Cu</td>
<td>copper</td>
</tr>
<tr>
<td>DLEC</td>
<td>distal lung epithelial cells</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>EIPA</td>
<td>5-(N-ethyl-N-isopropyl) amiloride</td>
</tr>
<tr>
<td>ENaC</td>
<td>epithelial Na⁺ channel</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GA</td>
<td>gestational age</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione (reduced)</td>
</tr>
<tr>
<td>GSSG</td>
<td>glutathione (oxidized)</td>
</tr>
<tr>
<td>H⁺</td>
<td>hydrogen ion</td>
</tr>
<tr>
<td>H₂O</td>
<td>water</td>
</tr>
<tr>
<td>HAPE</td>
<td>high altitude pulmonary edema</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balance salt solution</td>
</tr>
<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>IMCD</td>
<td>inner medullary collecting duct</td>
</tr>
</tbody>
</table>
\( I_{sc} \)  short circuit current
\( K^+ \)  potassium
\( kb \)  kilobase
\( kDa \)  kiloDalton
\( L^- \)  lipid alkyl radical
\( LO^- \)  lipid alkoxy radical
\( LOO^- \)  lipid peroxy radical
\( LPS \)  lipopolysaccharide (endotoxin)
\( MEM \)  minimal essential medium
\( M\phi \)  macrophage
\( Mg^{2+} \)  magnesium
\( Mn \)  manganese
\( MODS \)  multiple organ dysfunction syndrome
\( Na^+ \)  sodium
\( NAC \)  N-acetylcysteine
\( NADPH \)  reduced nicotinamide adenine dinucleotide phosphate
\( NMMA \)  \( N^G \)-monomethyl-L-arginine
\( NO \)  nitric oxide
\( NOS \)  nitric oxide synthase
\( NSC \)  non-selective cation
\( NTAb \)  anti-nitrotyrosine antibody
\( O_2 \)  oxygen
$O_2^-$ \hspace{1cm} \text{superoxide anion}

\cdot\text{OH} \hspace{1cm} \text{hydroxyl radical}

\text{PAWP} \hspace{1cm} \text{pulmonary artery wedge pressure}

\text{PBS} \hspace{1cm} \text{phosphate-buffered saline}

\text{PD} \hspace{1cm} \text{potential difference}

\text{PKA} \hspace{1cm} \text{protein kinase A}

\text{PKC} \hspace{1cm} \text{protein kinase C}

$P_{\text{Na}}/P_K$ \hspace{1cm} \text{sodium to potassium selectivity ratio}

$P_o$ \hspace{1cm} \text{open probability}

pS \hspace{1cm} \text{picoSiemens}

R \hspace{1cm} \text{resistance}

\text{RNA} \hspace{1cm} \text{ribonucleic acid}

\text{ROS} \hspace{1cm} \text{reactive oxygen species}

\text{RS-NO} \hspace{1cm} \text{S-nitrosothiol}

\text{SD} \hspace{1cm} \text{standard deviation}

\text{SIRS} \hspace{1cm} \text{systemic inflammatory response syndrome}

\text{SNAP} \hspace{1cm} \text{S-nitroso-N-acetylpenicillamine}

\text{SOD} \hspace{1cm} \text{superoxide dismutase}

\text{TNF} \hspace{1cm} \text{tumour necrosis factor}

\text{Zn} \hspace{1cm} \text{zinc}
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ABSTRACT

Our laboratory previously demonstrated that a vital component of the alveolar-capillary barrier, distal lung epithelial cell (DLEC) amiloride-sensitive Na⁺ transport, could be impaired by in vitro coculture with endotoxin (LPS)-stimulated alveolar macrophages (AM) through an L-arginine dependent mechanism. A similar, L-arginine independent impairment was seen resulting from exposure to the cell-free supernatant of LPS-stimulated AM. It was hypothesized that AM coculture might result in modulation of DLEC mRNA expression for the specific epithelial Na⁺ channel, rENaC. The role of peroxynitrite in the L-arginine dependent impairment of DLEC Na⁺ transport resulting from activated AM exposure was investigated. The ability of the thiol antioxidant N-acetylcysteine (NAC) to influence the changes in functional DLEC Na⁺ transport resulting from exposure to cell-free AM supernatant was also examined.

LPS-stimulated AM resulted in a significant decrease in mRNA expression for all subunits of rENaC. Further experiments showed that this process was not L-arginine dependent. Immunofluorescence studies using anti-nitrotyrosine antibodies failed to support an important role for peroxynitrite in the effect of activated AM on DLEC Na⁺ transport. Furthermore, it was shown that the impairment of DLEC Na⁺ transport resulting from exposure to LPS-stimulated AM supernatant was significantly modulated by NAC, which increases intracellular glutathione levels, but not by dithiothreitol (DTT), which does not specifically influence cell thiol levels.

These studies, then, elucidate mechanisms by which activated AM impair alveolar epithelial barrier function in an in vitro model of acute lung injury.
INTRODUCTION AND LITERATURE REVIEW

1.1 Clinical and Pathological Features of Acute Lung Injury

1.1.1 Clinical Considerations

The acute respiratory distress syndrome (ARDS) is a significant cause of mortality among critically ill patients (21). Despite decades of basic and clinical research efforts, fundamental aspects of the pathophysiology of ARDS remain poorly understood and virtually no impact has been made on the underlying grave prognosis among patients with the fully established syndrome, whose mortality is over 50% in most series (see below).

Although severe pulmonary failure was recognized among casualties of the battlefields of World War I (200), the first formal description of ARDS appeared in 1967 when Ashbaugh and colleagues reported their experience with 12 patients in whom a variety of insults resulted in a similar syndrome of respiratory distress (4). Characterized by "severe dyspnea, tachypnea, cyanosis refractory to oxygen therapy, loss of lung compliance, and diffuse alveolar infiltration seen on chest x-ray", it resulted in a mortality rate of 58% despite aggressive therapy, including mechanical ventilation. Its name was originally chosen because it shared many of the clinical characteristics of the then recently described respiratory distress syndrome (RDS) occurring in premature infants. Subsequent work has shown that the initial insult in newborns is different than that in ARDS. The body of literature regarding ARDS has grown immensely since 1967 but this basic clinical description has largely endured, as has the high mortality rate.
Numerous attempts to expand or even clarify the fundamental definitions of ARDS in the intervening years have resulted in a certain degree of confusion, making interpretation of clinical and epidemiological studies somewhat difficult. In view of this, a consensus conference on ARDS was recently convened to summarize opinion and formulate recommendations regarding these basic issues (21). Emphasized in the report's definitions was the concept that the pulmonary derangements resulting from varied predisposing events represent a spectrum of dysfunction termed “acute lung injury” (ALI), with ARDS being the most severe expression of this process (Table 1).

<table>
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<td><strong>Timing</strong></td>
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<td>ARDS Criteria</td>
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$P_aO_2/F_iO_2$ - systemic arterial partial pressure of oxygen to fraction of inspired oxygen ratio
PAWP - pulmonary arterial wedge pressure

*adapted from (21)

The specific clinical criteria listed reflect the underlying pathophysiology of ALI, the basis of which is an injury and inflammation to the alveolar-capillary barrier which normally maintains an essentially fluid-free alveolar space. The failure of this structure, which consists of the pulmonary capillary endothelium, the alveolar epithelium and a
thin layer of intervening connective tissue, leads to “high permeability” pulmonary edema with resulting profound and progressive abnormalities of gas exchange. The onset of these changes usually occurs less than 72 hours after the inciting event (110), and may occur in less than 6 hours in some severe settings, particularly sepsis (74,156). The bilateral radiological changes reflect the diffuse nature of the process. In the seminal report by Ashbaugh and colleagues, the 12 involved patients, while sharing these common, severe pulmonary derangements, differed in their primary diagnoses. Eight of these patients had suffered traumatic injuries, 4 had presumed viral pneumonia and 1 had been diagnosed with pancreatitis. What was recognized then, therefore, was that varied insults, both direct and distant, could lead to a common syndrome of ALI (Table 2). What these disparate processes have in common

<table>
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<tr>
<th>Direct Injury</th>
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<tr>
<td>Aspiration</td>
<td>Sepsis syndrome</td>
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<tr>
<td>Diffuse pulmonary infection</td>
<td>Severe nonthoracic trauma</td>
</tr>
<tr>
<td>Near drowning</td>
<td>Pancreatititis</td>
</tr>
<tr>
<td>Toxic inhalation</td>
<td>Cardiopulmonary bypass (rare)</td>
</tr>
<tr>
<td>Lung contusion</td>
<td>Emergency hypertransfusion</td>
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</table>

*adapted from (21)*

is their ability to initiate a profound and unbridled activation of normal inflammatory mechanisms, which has come to be known as the systemic inflammatory response syndrome (SIRS) (26). In this way, a given insult can result in a cascade of inflammatory events, both cellular and humoral (64), leading to distant tissue injury and, ultimately, distant organ damage and failure. Recently, this has been termed “multiple
organ dysfunction syndrome" (MODS) (26). ARDS almost always precedes MODS (39,64), and the importance of this sequence of events clinically is reflected by the fact that mortality among patients with ARDS is clearly increased by the superimposition of non-pulmonary organ failure (129,168,229,247). In fact, a number of studies have shown that it is uncommon for ARDS patients to succumb from irreversible pulmonary failure with MODS implicated as a principal cause of death (168,169).

Prominent among the predisposing causes of ARDS (see above) is infection and the sepsis syndrome. Although recognition of the importance of non-infectious initiating events has increased recently, most studies implicate infection as the commonest antecedent event. Sepsis represents a poor prognostic indicator and is the most common associated cause of death in patients with MODS (73,108,129,156,168,229,247). Support for these clinical observations comes from laboratory studies showing that infection can produce ARDS (169), including models involving live bacterial or endotoxin infusions. Endotoxin, or lipopolysaccharide (LPS), is a complex glycolipid found in the cell walls of gram negative bacteria, which are common initiators of sepsis-associated lung injury (69). In animal models, LPS infusion results in increased pulmonary vascular resistance, endothelial cell injury, and attenuation of hypoxic vasoconstriction (148), each of which is characteristic of patients with ARDS. These effects of endotoxin, while mediated in part by direct toxic events such as injury to lung capillary endothelium (164), result largely from a complex interplay with the host immune system, involving activation of inflammatory cells (including macrophages and neutrophils) and release of a wide range of soluble
mediators (including cytokines, proteases, pro-oxidants, lipid-derived mediators, complement, clotting factors and nitric oxide) (147). Reflecting the varied and multifactorial nature of this process are the disappointing results of trials using anti-endotoxin monoclonal antibodies in sepsis (148,160).

As mentioned above, epidemiological studies regarding ARDS have been limited by varying definitions of the syndrome itself. A more recent estimate of the incidence of the syndrome, arising from a population-based study, indicates a rate in the range of 1.5 - 3.5 per 100 000 (264). This contrasts to original estimates of approximately 75 per 100,000. This apparent change in incidence rates may reflect better initial care of patients suffering predisposing insults and, perhaps, the fact that earlier figures represent overestimates.

Virtually all studies, however, identify a mortality rate that exceeds 50% (73,74,108,110,156,168,229,247) among patients who do develop ARDS, consistent with Ashbaugh and colleagues' reported rate of 58%. Despite almost 3 decades of improvement in the understanding of the pathophysiology of acute lung injury as well as increasingly sophisticated intensive care, ARDS remains a devastating problem in critically ill patients. Indeed, although supportive care for these patients is clearly vital, iatrogenic factors, notably positive pressure ventilation and supplemental oxygen therapy, are well recognized to contribute to the progression of ALI. Efforts continue in order to better understand the basic mechanisms resulting in the development of and the recovery from ALI. New knowledge may be applied to therapeutic or preventative strategies. In this regard, some attention has been focused on the alveolar-capillary
barrier itself, and specifically alveolar fluid clearance mechanisms which appear to be adversely affected in patients with ARDS (158).

1.1.2 Pathology of ALI and ARDS

The pathological features of ALI are consistent with a severe inflammatory organ injury, and, in keeping with the clinical characteristics, are generally similar regardless of the inciting insult. This is not surprising given the concept of ALI as an end organ manifestation of SIRS, characterized by an underlying injury to the alveolar-capillary barrier. The acute phase of ALI is dominated by alveolar flooding as a result of this derangement with the consequent clinical manifestations described above. As expected, given the spectrum of dysfunction inherent in the definition of ALI, the extent of injury and its subsequent course is variable. Patchy involvement, seen both on a pathologic and an imaging basis (81), is a common feature. In some patients, especially those free of non-pulmonary organ failure, the acute injury may resolve quickly. Often, however, a progression of the syndrome is seen that reflects ongoing inflammation and tissue damage as well as reparative mechanisms which can ultimately lead, in those who survive, to interstitial and intra-alveolar fibrosis. Given this temporal progression of pathological changes, ALI can be divided into three, albeit overlapping, phases: the exudative, proliferative and fibrotic phases (257).

Exudative Phase This is the acute phase of ALI, encompassing approximately the first week following the onset of respiratory failure (126), and is essentially
characterized by the "high-permeability" pulmonary edema which has become one of the hallmark features of ARDS. Grossly congested, fluid-filled lungs reflect the underlying characteristics of this phase, including capillary congestion and focal neutrophil (polymorphonuclear leukocyte, PMN) aggregation, interstitial and alveolar edema and intra-alveolar hemorrhage (257). Occlusion of small, extra-alveolar arteries and veins is seen and is due largely to thromboemboli, both local and systemic, identified in as many as 95% of patients at postmortem lung examination (256). This has also been shown in vivo by selective pulmonary angiography (88). Other contributing factors may include compression by perivascular hemorrhage and edema, and, later, by diffuse necrotizing vasculitis and vessel wall sclerosis (6). This vascular occlusion can contribute to the pulmonary hypertension and ventilation-perfusion mismatching seen in ARDS. Although interstitial exudation results from microscopic changes involving the endothelial component of the alveolar-capillary barrier, including cell swelling, widening of intercellular junctions and an abundance of pinocytotic vesicles (257), these are generally less striking than those of the alveolar epithelium (7). Although alveolar type II epithelial (ATII)-like cells are generally present, extensive necrosis of the primary alveolar lining cell, the alveolar type I epithelial (ATI) cell, which covers most of the alveolar space, leads to a denuded alveolar basement membrane (257). Loss of this vital epithelial barrier results in the intra-alveolar accumulation of cells, including small numbers of PMN, and plasma proteins. The latter, comprised largely of immunoglobulin, fibrin(ogen) and complement (75), condense with cellular debris to form hyaline membranes (7), which are non-specific indicators of lung injury.
Hyaline membranes primarily line the alveolar ducts at the entrance to alveoli, but will adhere to any area of epithelial denudation (6). This protein-rich exudate reflects the increased pulmonary endothelial and epithelial permeability to protein and fluid, a hallmark of ALI. As the interstitial fluid floods the alveolus, a profound impairment of gas exchange develops.

_Proliferative Phase_ This phase of ALI can be viewed as one in which the various materials accumulated in the exudative phase are organized and the machinery put in place for either the repair or subsequent fibrotic phase. In individuals who survive the acute phase, this reparative process, when poorly regulated, can lead to permanent changes in the lung's specific compliance and alveolar volume-corrected diffusion characteristics, derangements which have been identified as a poor prognostic indicators (132).

While the exudative phase primarily involves phagocytic cells, the proliferative phase is dominated by two distinct cell types; the ATII cell and the fibroblast. The onset of this phase features the proliferation of ATII-like cells which regenerate and cover areas of the basement membrane denuded in the exudative phase. This can occur as early as the third day following the onset of ARDS (257). These cells have the ability to ultimately differentiate into ATI epithelial cells (1). Fibroblasts, meanwhile, proliferate in the interstitium and migrate into the alveolar spaces and regions of fibrin deposition, converting previous exudate into granulation tissue and, eventually, fibrous tissue, through collagen deposition (75). ATII epithelial cells epithelialize the surface of this
organizing granulation tissue, converting it into interstitial-like tissue (257). Fibroblasts also deposit connective tissue in the alveolar wall with consequent widening of the alveolar septa (7). In addition, organizing fibrin and hyperplastic epithelium, combined with possible surfactant abnormalities, can cause loss of volume with ultimate permanent collapse of alveolar walls. The end result of these processes is a progressive increase in interstitial tissue and intra-alveolar fibrosis with an irreversible loss of specific lung compliance and obliteration of airspaces (257).

**Fibrotic Phase** The changes seen in this phase represent the full expression of the processes occurring in the proliferative phase. In ventilator-dependent patients surviving for weeks, lung architecture can be completely distorted by fibrosis. Grossly, lung parenchyma is pale and alternating areas of microcystic airspaces are seen (257). Microscopically, alveolar septa and airspace walls are thickened by minimally cellular, collagenous connective tissue and surviving airspaces are irregularly enlarged. In keeping with this, there is a progressive increase in total lung collagen with increasing duration of disease (283). Extensive vascular remodeling also occurs in the late proliferative and fibrotic phases. Reaction to endothelial injury, termed fibrointimal proliferation (257), leads to a reduction of vascular luminal area.

The normal alveolar septa are thin structures, averaging 2.2 μm in thickness (257), and are well suited for gas exchange with the closely apposed capillaries. Progression of ALI, however, results in reduced surface area and increased thickness of the alveolar septa as well as profound effects on the lung’s vascular architecture.
The potentially devastating functional sequelae of these processes are well illustrated in data obtained from lungs of patients who died several weeks after the onset of ARDS (6). The morphometrically estimated diffusion capacity, an index of the lung's gas exchange capability, was reduced to approximately 10% of normal.

Clearly, then, ARDS is characterized pathologically by profound and potentially progressive damage to the alveolar-capillary membrane. Even among patients who survive the acute or early proliferative phases of ALI, a proportion will suffer permanent pulmonary fibrosis, a significant cause of long term morbidity and mortality (161).

1.2 Mediators of Acute Lung Injury
1.2.1 Phagocyte Mediators of Acute Lung Injury

Although ALI can result from direct toxic injury to the lung parenchyma, the more common pathway involves the activation of leukocytes and the subsequent release of a variety of mediators, leading to the spectrum of injury described above. Leukocytes are, under normal circumstances, involved in host defense. In keeping with the fundamental pathogenesis of SIRS and MODS, the uncontrolled and widespread activation of these inflammatory cells can characterize the functional and structural derangements of ALI. Potentially injurious leukocyte products include cytokines (e.g. tumour necrosis factor, interleukins), proteolytic enzymes (e.g. lysozyme, collagenases, elastases), biologically active lipids (e.g. arachidonic acid metabolites) and reactive oxygen species (e.g. superoxide, hydroxyl radical, hydrogen peroxide, hypochlorous...
acid) (228). Interest has been largely focused on the most abundant leukocyte in this regard, the PMN, and a great deal of evidence supports a primary role for this inflammatory cell type in the pathogenesis of ARDS. The role of the macrophage (Mφ), another leukocyte capable of the release of all the mediators listed above and the only resident inflammatory cell in the alveolus (67), has likely been underestimated.

The alveolar macrophage (AM) has long been recognized as a source of chemoattractant factors important in the process of neutrophil sequestration and migration into the lung. A conceptual framework has evolved in which an injurious insult, such as the release of endotoxin in gram negative infection, acts to both directly activate PMN and to stimulate the release of chemoattractant factors from resident lung cells, including AM (236). These products then act to promote the complex process of PMN accumulation within the lung (see below). The characterization of a specific chemotactic cytokine, IL-8, released by AM in the setting of lung inflammation further supports this model (8). In a study involving patients at risk for ALI, elevated levels of bronchoalveolar lavage (BAL) fluid IL-8 correlated with the subsequent development of ARDS (63). Immunohistochemical analysis of cells from these BAL samples confirmed that AM represent an important source of IL-8. Although attention has generally focused on this aspect of AM function in ALI, evidence exists, as discussed below, suggesting a more direct role for AM in the pathologic changes discussed above.
1.2.1.1 The PMN in Acute Lung Injury

Pathologically, ALI is characterized by increased intravascular and lung parenchymal sequestration of PMN (see above). Although the normal lung has large numbers of PMN in its vascular bed, they are virtually absent its extra-vascular spaces (228). PMN-mediated lung injury involves the following sequence of events (65): a) delivery of PMN to the lung, b) margination or sequestration within the pulmonary microvasculature, c) adhesion to the pulmonary capillary endothelial cells, and d) emigration out of the vascular space.

Delivery of PMN to the lung in the setting of an inflammatory insult is facilitated by the fact that the pulmonary vasculature receives the entire cardiac output, such that all circulating PMN must eventually pass through the lung. In addition, approximately two thirds of PMN within the vasculature are non-circulating, or marginated (104). Mechanical factors related to the pulmonary microvasculature dictate that the large majority of marginated leukocytes already exist within the lung (65), resulting in a significant number of PMN resting in close apposition to the capillary endothelium even before activation occurs.

Exposure of PMN to various inflammatory mediators, including endotoxin, results in a complex series of events leading to the sequence outlined above. First, increased stiffness or rigidity of activated PMN (279), related to cytoskeletal changes, impair their ability to pass through the pulmonary microvasculature, resulting in their retention, or sequestration, largely at the level of the capillaries (60). This is followed by specific, membrane receptor-ligand-mediated adhesion between these retained cells and the
capillary endothelium. Three broad categories of adhesion molecules contributing to
cell-cell interactions have been described (65): (i) the immunoglobulin superfamily
(intercellular adhesion molecule or ICAM), expressed by endothelial cells, (ii) the
integrin family, including the CD11/CD18 complex (β2-integrins), expressed by
leukocytes, and (iii) the selectins, which are expressed on both PMN (L-selectin) and
endothelial cells (P- and E-selectin). The expression and activity of these molecules
are specifically regulated in inflammation. This includes an increase in the
adhesiveness of the PMN β2-integrins and increased endothelial cell membrane
expression of the ICAM molecules and the selectins in response to a number of
inflammatory stimuli (232). The resulting high affinity interactions between leukocytes
and endothelial cells lead to prolonged retention within the pulmonary microvasculature
and eventual movement of PMN between endothelial cells (170), into the interstitial
space and, ultimately, the alveolar space. Indeed, the crucial role of PMN adhesion
and subsequent migration in this process is reflected by studies using in vivo models of
ALI, in which significant attenuation of PMN influx results from treatment with antibodies
directed against the adhesion molecules described above (tables 3-5). The process of
adhesion also serves to increase PMN responsiveness to subsequent stimuli, in a
process called “priming” (65).
Table 3. Contribution of selectins and their ligands to lung neutrophil sequestration in ALI

<table>
<thead>
<tr>
<th>Selectins</th>
<th>Species</th>
<th>Model</th>
<th>Intervention</th>
<th>Ab clone</th>
<th>% reduction in lung PMN influx*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-selectin</td>
<td>rat</td>
<td>Thermal injury</td>
<td>Anti P-selectin mAb</td>
<td>PB1.3</td>
<td>35 (176)</td>
<td></td>
</tr>
<tr>
<td>P-selectin</td>
<td>rat</td>
<td>Cobra venom factor</td>
<td>Anti P-selectin mAb</td>
<td>PB1.3</td>
<td>35 (174)</td>
<td></td>
</tr>
<tr>
<td>P-selectin</td>
<td>rat</td>
<td>Extremity reperfusion injury</td>
<td>Anti P-selectin mAb</td>
<td>PB1.3</td>
<td>89 (223)</td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td>rat</td>
<td>Thermal injury</td>
<td>Anti E-selectin mAb</td>
<td>CL-3</td>
<td>70 (176)</td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td>rat</td>
<td>Extremity reperfusion injury</td>
<td>Anti E-selectin mAb</td>
<td>CL-3</td>
<td>67 (223)</td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td>rat</td>
<td>Intestinal reperfusion injury</td>
<td>Anti E-selectin mAb</td>
<td>BBIG-E5</td>
<td>96 (2)</td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td>rat</td>
<td>Intratracheal lipopolysaccharide</td>
<td>Anti E-selectin mAb</td>
<td>A3 or M4</td>
<td>A3-51 (BAL fluid PMN) M4-66 (BAL fluid PMN) (261)</td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td>rat</td>
<td>Intratracheal IL-1b</td>
<td>Anti E-selectin mAb</td>
<td>M4</td>
<td>65 (BAL fluid PMN) (261)</td>
<td></td>
</tr>
<tr>
<td>L-selectin</td>
<td>rat</td>
<td>Thermal injury</td>
<td>Anti L-selectin mAb</td>
<td>HRL-1</td>
<td>58 (176)</td>
<td></td>
</tr>
<tr>
<td>L-selectin</td>
<td>rat</td>
<td>Extremity reperfusion injury</td>
<td>Anti L-selectin mAb</td>
<td>Not specified</td>
<td>53 (223)</td>
<td></td>
</tr>
<tr>
<td>L-selectin</td>
<td>rat</td>
<td>Lung immune complex deposition</td>
<td>Anti-L-selectin mAb</td>
<td>HRL1</td>
<td>0 (BAL fluid PMN) (177)</td>
<td></td>
</tr>
<tr>
<td>E,L-selectin</td>
<td>pig</td>
<td>Pseudomonas bacteremia</td>
<td>Anti E, anti L-selectin mAb</td>
<td>EL-246</td>
<td>69, 80 (%PMN in BAL fluid) (209)</td>
<td></td>
</tr>
<tr>
<td>Nonspecific</td>
<td>rat</td>
<td>Cobra venom factor</td>
<td>Sialyl Lewis* oligosaccharides</td>
<td>40</td>
<td>40 (173)</td>
<td></td>
</tr>
<tr>
<td>Nonspecific</td>
<td>rat</td>
<td>Lung IgG immune complex deposition</td>
<td>Sialyl Lewis* oligosaccharides</td>
<td>46</td>
<td>46 (172)</td>
<td></td>
</tr>
<tr>
<td>Nonspecific</td>
<td>rat</td>
<td>Extremity reperfusion injury</td>
<td>Sialyl Lewis* oligosaccharides</td>
<td>65</td>
<td>65 (223)</td>
<td></td>
</tr>
</tbody>
</table>

*As measured by myeloperoxidase activity unless otherwise specified.

Table 4. Contribution of ICAM-1 to lung neutrophil sequestration in ALI

<table>
<thead>
<tr>
<th>Ig superfamily</th>
<th>Species</th>
<th>Model</th>
<th>Intervention</th>
<th>Ab clone</th>
<th>% reduction in lung PMN influx*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1</td>
<td>mouse</td>
<td>Systemic endotoxemia</td>
<td>ICAM-1 knockout</td>
<td></td>
<td>no difference (histological evaluation)</td>
<td>(280)</td>
</tr>
<tr>
<td>ICAM-1/P-selectin</td>
<td>mouse</td>
<td>Intrabronchial S. pneumoniae</td>
<td>P-selectin/ICAM-1 double knockout</td>
<td></td>
<td>0</td>
<td>(32)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>rat</td>
<td>Extremity reperfusion injury</td>
<td>Anti-ICAM-1 mAb</td>
<td>1A29</td>
<td>30 (222)</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>rat</td>
<td>Intestinal reperfusion injury</td>
<td>Anti-ICAM-1 mAb</td>
<td>1A29</td>
<td>98 (237)</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>rat</td>
<td>Lung immune complex deposition</td>
<td>Anti-ICAM-1 mAb</td>
<td>1A29</td>
<td>44 (179)</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>rat</td>
<td>Cobra venom factor</td>
<td>Anti-ICAM-1 mAb</td>
<td>1A29</td>
<td>64 (175)</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>rat</td>
<td>Thermal injury</td>
<td>Anti-ICAM-1 mAb</td>
<td>1A29</td>
<td>78 (176)</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>rat</td>
<td>Lung immune complex deposition</td>
<td>Anti-ICAM-1 mAb (intratracheal)</td>
<td>1A29</td>
<td>63 (BAL fluid PMN) (177)</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>rabbit</td>
<td>Lung reperfusion injury</td>
<td>Anti-ICAM-1 mAb</td>
<td>RR1/1</td>
<td>52 (106)</td>
<td></td>
</tr>
</tbody>
</table>

*As measured by myeloperoxidase activity unless otherwise specified.
Migration of PMN within the lung parenchyma is likely regulated by a
chemotactic gradient resulting, at least in part, from products released by resident cells,
such as AM, endothelial cells and lung fibroblasts (65). Once this process has
occurred, there exists ample evidence for the importance of PMN-mediated events in
ALI.

The release of toxic mediators from PMN is known to occur following stimulation
with a number of agents, including endotoxin (231). The importance of PMN has been
demonstrated in animal models of ARDS, including those mimicking infection with the
administration of endotoxin (31) and live bacteria (267,278). In patients with ARDS,
BAL fluid contains significantly higher numbers of PMN as compared to intubated, non-

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Table 5. Contribution of integrins to lung neutrophil sequestration in ALI

<table>
<thead>
<tr>
<th>Integrins</th>
<th>Species</th>
<th>Model</th>
<th>Intervention</th>
<th>Ab clone</th>
<th>% reduction in lung PMN influx*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD18</td>
<td>rabbit</td>
<td>Intrabronchial C5a</td>
<td>Anti-CD18 mAb</td>
<td>60.3</td>
<td>43 (airspace 111In-labeled PMN)</td>
<td>(100)</td>
</tr>
<tr>
<td>CD18</td>
<td>rabbit</td>
<td>Intrabronchial IL-1</td>
<td>Anti-CD18 mAb</td>
<td>60.3</td>
<td>66 (airspace 111In-labeled PMN)</td>
<td>(100)</td>
</tr>
<tr>
<td>CD18</td>
<td>pig</td>
<td>Pseudomonas bacteremia</td>
<td>Anti-CD18 mAb</td>
<td>60.3</td>
<td>60 (PMN in BAL, fluid)</td>
<td>(267)</td>
</tr>
<tr>
<td>CD18</td>
<td>rabbit</td>
<td>Intratracheal S. pneumoniae</td>
<td>Anti-CD18 mAb</td>
<td>60.3</td>
<td>66 (BAL fluid PMN)</td>
<td>(276)</td>
</tr>
<tr>
<td>CD18</td>
<td>rabbit</td>
<td>Intratracheal E. coli or lipopolysaccharide</td>
<td>Anti-CD18 mAb</td>
<td>60.3</td>
<td>80 (BAL fluid PMN)</td>
<td>(276)</td>
</tr>
<tr>
<td>CD18</td>
<td>rabbit</td>
<td>Intestinal reperfusion injury</td>
<td>Anti-CD18 mAb</td>
<td>R15.7</td>
<td>76</td>
<td>(102)</td>
</tr>
<tr>
<td>CD18</td>
<td>rabbit</td>
<td>Lung reperfusion injury</td>
<td>Anti-CD18 mAb</td>
<td>IB4</td>
<td>64</td>
<td>(106)</td>
</tr>
<tr>
<td>CD18</td>
<td>rabbit</td>
<td>Zymosan activated plasma</td>
<td>Anti-CD18 mAb</td>
<td>60.3</td>
<td>100 (total lung 111Cr labeled PMN)</td>
<td>(59)</td>
</tr>
<tr>
<td>CD18</td>
<td>rabbit</td>
<td>Extremity reperfusion injury</td>
<td>Anti-CD18 mAb</td>
<td>CL-26</td>
<td>66</td>
<td>(222)</td>
</tr>
<tr>
<td>CD18</td>
<td>rabbit</td>
<td>Thermal injury</td>
<td>Anti CD18 mAb</td>
<td>CL-26</td>
<td>80</td>
<td>(175)</td>
</tr>
<tr>
<td>CD11a</td>
<td>rat</td>
<td>Lung immune complex deposition</td>
<td>Anti-CD11a mAb (intratracheal)</td>
<td>WT-1</td>
<td>47 (BAL fluid PMN)</td>
<td>(179)</td>
</tr>
<tr>
<td>CD11a</td>
<td>rat</td>
<td>Lung immune complex deposition</td>
<td>Anti-CD11a mAb (intratracheal)</td>
<td>WT-1</td>
<td>47 (BAL fluid PMN)</td>
<td>(179)</td>
</tr>
<tr>
<td>CD11a</td>
<td>rat</td>
<td>Lung immune complex deposition</td>
<td>Anti-CD11a mAb (intratracheal)</td>
<td>WT-1</td>
<td>47 (BAL fluid PMN)</td>
<td>(179)</td>
</tr>
<tr>
<td>CD11b</td>
<td>rat</td>
<td>Cobra venom factor</td>
<td>Anti-CD11b mAb</td>
<td>186</td>
<td>51</td>
<td>(175)</td>
</tr>
<tr>
<td>CD11b</td>
<td>rat</td>
<td>Cobra venom factor</td>
<td>Anti-CD11b mAb</td>
<td>186</td>
<td>58</td>
<td>(175)</td>
</tr>
<tr>
<td>CD11a</td>
<td>rat</td>
<td>Thermal injury</td>
<td>Anti CD11a mAb</td>
<td>WT-1</td>
<td>34</td>
<td>(176)</td>
</tr>
<tr>
<td>CD11a</td>
<td>rat</td>
<td>Extremity reperfusion injury</td>
<td>Anti-CD11a mAb</td>
<td>WT-1</td>
<td>34</td>
<td>(176)</td>
</tr>
<tr>
<td>CD11b</td>
<td>rat</td>
<td>Extremity reperfusion injury</td>
<td>Anti-CD11b mAb</td>
<td>186</td>
<td>47</td>
<td>(176)</td>
</tr>
<tr>
<td>CD11b</td>
<td>rat</td>
<td>Lung immune complex deposition</td>
<td>Anti-CD11b mAb (intratracheal)</td>
<td>186</td>
<td>78</td>
<td>(177)</td>
</tr>
<tr>
<td>CD11b</td>
<td>rat</td>
<td>Thermal injury</td>
<td>Anti CD11b mAb</td>
<td>not specified</td>
<td>60</td>
<td>(176)</td>
</tr>
<tr>
<td>CD11b</td>
<td>rat</td>
<td>Lung immune complex deposition</td>
<td>Anti-CD11b mAb</td>
<td>186</td>
<td>34</td>
<td>(179)</td>
</tr>
</tbody>
</table>

*As measured by myeloperoxidase activity unless otherwise specified.
ARDS patients (271). In this setting, PMN represented, in fact, the most abundant cell type. The relative percentage of PMN has been correlated with the severity of gas exchange abnormalities as well as lung protein permeability (271). A relationship between this and mortality was identified in patients with sepsis-related ARDS (246). Analysis of BAL fluid from patients with ALI reveals not only an increase in cell numbers but also evidence of PMN-derived toxic products, including PMN elastase and collagenase (271).

PMN, then, are likely the major phagocyte mediators of ALI. Several observations, however, implicate a role for other cell types, including the fact that neutropenic patients can develop typical ARDS (27,159,192). In addition, the prevention of the PMN-mediated lung injury, either through the experimental depletion of PMN (103), or the prevention of PMN adhesion to endothelial cells with anti-CD18 antibody (267) (see above), attenuates, but does not completely prevent, ALI in animal models of sepsis.

1.2.1.2 The Macrophage in Acute Lung Injury

Mφ exist in many organs and tissues as resident inflammatory cells and, as such, are components of the reticuloendothelial system. The majority of AM represent migrated and differentiated circulating monocytes (25) which arise from bone marrow precursors (253). AM are the only resident inflammatory cells within the alveolar space (67) and represent approximately 10% of total human lung cells under normal circumstances (270). Although some investigators question the ability of Mφ to
replicate within the lung (228), an increase in the number of AM in the setting of lung inflammation has been documented, resulting both from monocyte migration and local replication (24).

With regard to acute lung injury, emphasis has been largely placed on the role of the AM in the resolution of inflammation. The vital role of Mφ in dermal wound healing has been long recognized (137). Views regarding the role of Mφ in ALI have evolved from a conceptual model characterized by a largely PMN-dependent acute phase, characterized by PMN accumulation and predominance in the airspace within hours, followed by a gradual accumulation of AM (101,246). AM then remove airspace debris, which involves the ingestion of PMN (181) as well as the clearance of proteinaceous material (101). In keeping with this, AM isolated from hamsters in the latter stages of ALI have been shown to acquire PMN enzyme activity, consistent with the phagocytosis of these cells (53). This same study demonstrated increased fibrinolytic activity in isolated AM, which might facilitate airspace fibrin removal.

Compelling evidence also exists, however, implicating the Mφ in the initiation and propagation of ALI, beyond their role in the chemoattraction of PMN (29,65,163,242). A process similar to that for PMN lung accumulation in sepsis is suggested by a study involving endotoxin stimulation of monocytes, both in vitro and in vivo (61). Rapid retention of monocytes in the lungs of rabbits injected with LPS was demonstrated, a process apparently related to enhanced monocyte stiffness and cytoskeletal modulation. LPS treatment also resulted in adhesion of monocytes to human endothelial cell monolayers through CD-18-dependent and independent mechanisms.
CD-18-dependent adhesive mechanisms were implicated in prolonged monocyte retention in LPS-injected rabbits. This study, then, provides evidence for an early accumulation of monocytes in the lung in response to an inflammatory insult. Combined with resident AM, this could contribute to Mφ-mediated lung injury. It is known that Mφ are capable of releasing a spectrum of products which are injurious to tissues (180), similar to PMN. For example, in an animal model of sepsis in which rats underwent laparotomy with cecal ligation and puncture, AM obtained by BAL demonstrated increased adherence and spreading ability, as well as enhanced release of superoxide anion (O$_2^-$), a reactive oxygen species (ROS), and lysosomal enzymes, when stimulated in vitro (86). This is another example of priming, in which a given stimulus results in increased cell activity in response to a subsequent stimulus (62). The time course of these changes in AM function paralleled the development of ALI in the rats, as reflected in BAL fluid protein concentrations. Immune-complex-mediated lung injury in rats resulted in an early increase in lung Mφ that paralleled the accumulation of PMN (30). AM recovered from these rats were primed to release increased levels of O$_2^-$ in response to in vitro stimulation. Intra-tracheal instillation of phorbol myristate acetate resulted in complement-derived, PMN-independent injury that was also associated with AM oxidant release (123). AM obtained by BAL from mice following severe hemorrhage and resuscitation, which resulted in histologic evidence of ALI, showed significantly increased mRNA expression of a number of inflammatory cytokines (226).
Studies in humans support the concept of the Mϕ as an important cellular mediator in ALI. Intravenous endotoxin administration to normal human volunteers resulted in the development of constitutional symptoms and fever (233). AM recovered from BAL fluid were shown to be primed to release enhanced levels of interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α) and prostaglandin E₂ (PGE₂). Extending this to patients with ALI, Jacobs and colleagues studied AM from patients with ARDS and compared them to those recovered from patients with pneumonia and from healthy volunteers (119). AM from subjects with ARDS released more IL-1 as compared to those from subjects in all other groups, including patients with severe pneumonia requiring mechanical ventilation. Treatment of AM from ARDS patients in vitro with LPS also resulted in a significantly greater increase in IL-1 release, again demonstrating a priming effect. These data indicate a Mϕ activation that may be unique to ARDS, not seen in the setting of a more localized inflammatory insult such as pneumonia, even if such a process might ultimately lead to ARDS. Finally, ALI in humans is not necessarily a PMN-dependent process. As indicated above, severely neutropenic patients can develop typical ARDS (27,159,192). Furthermore, high-altitude pulmonary edema (HAPE) is a form of ALI that appears not to involve a significant intra-alveolar accumulation of PMN. HAPE is characterized by high permeability pulmonary edema that develops in a small proportion of individuals at high altitude (220). Analysis of BAL fluid from such individuals revealed a significant rise in protein content, as compared to BAL fluid from normal subjects, and from those with acute mountain sickness (AMS), a milder form of altitude-associated lung dysfunction (219,220). Measurement of BAL fluid
cell count revealed a significant rise in leukocyte numbers, consisting primarily of an increase in AM.

1.2.2 Specific Humoral Mediators of Acute Lung Injury

1.2.2.1 Nitric Oxide in Acute Lung Injury

Among the products secreted by activated Mφ is NO, a substance that has been the focus of a great deal of recent attention. In the lung, NO from a variety of sources is thought to mediate a number of essential bioactivities, including vasodilatation, bronchodilation, neurotransmission, and host defense against infection and tumour growth (13,79,125,282). The assertion has been made, in fact, "that virtually every mammalian cell is under the influence of NO" (216). NO is synthesized in a variety of cells through the action of NO-synthase (NOS) on the terminal guanidino nitrogen atom of L-arginine (figure 1). NOS can therefore be inhibited by N\textsuperscript{G} monomethyl-L-arginine, an amino acid analogue that competes with arginine (125). NOS is

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{nitric_oxide_synthetic_pathway.png}
\caption{Nitric Oxide Synthetic Pathway}
\end{figure}
found in a Ca$^{2+}$-dependent, constitutive form (cNOS) in vascular endothelium, platelets, PMN and neural tissue (125). In other cell types, including Mφ (124), NOS exists in an inducible, Ca$^{2+}$-independent subtype (iNOS). Regulation of iNOS by inflammatory mediators in a number of tissues and cell types has been shown. Whole lung iNOS mRNA expression was increased in response to intraperitoneal (145) and intravenous (277) endotoxin injection in rats. Within the lung, induction of iNOS in rat AM in response to endotoxin and cytokines, including interferon-γ (IFN-γ), has been demonstrated (124). Recently, immunochemical detection confirmed that iNOS exists in stimulated human AM (258). Furthermore, cultured ATII epithelial cells exposed to a mixture of cytokines with or without LPS were shown to produce significant amounts of NO as a result of increased expression of iNOS (90,205). More recent work from our laboratory has shown that fetal DLEC iNOS expression could be induced by a soluble factor from LPS-stimulated AM, the exact nature of which is unknown (56).

Although these findings could be consistent with a role for NO in the host response to inflammatory insults, evidence exists implicating NO as a mediator of lung injury itself. NO has been shown to play an important role in ALI resulting from inhaled irritants (114,205) and immune complex injury (171,178). An NO-mediated impairment of fetal DLEC Na$^{+}$ transport has been demonstrated as a result of coculture with endotoxin-stimulated AM (48).

NO has a very short half-life of between 0.1 and 5 seconds in physiological systems, resulting largely from oxidation or interaction with transition metals, especially iron (79). The latter includes the reaction of NO with the heme moiety in hemoglobin.
Reaction between NO and superoxide results in the formation of peroxynitrite, a very potent ROS, which has been shown to induce lipid peroxidation (206) and the oxidation of DNA bases (15) and cell thiols (207). Peroxynitrite can also undergo further reaction with the formation of the extremely unstable ROS, hydroxyl radical (OH·), or a ROS with similar properties (79). Evidence exists supporting a potential role for peroxynitrite formation in the pathogenesis of ALI (see discussion).

The principal mechanism by which NO exerts biological effects is through the interaction of its derivatives with transition metals and thiol-containing proteins. This includes the activation by NO of guanylate cyclase, an enzyme which mediates an increase in cellular cGMP levels, and elicits many target cell responses (125). This activation results from the binding of NO with the enzyme’s iron-containing heme moiety, and is possibly associated with a structural change in the protein (239).

The reaction of NO-derived species with intracellular thiol compounds represents an important means by which NO acts in biological systems, due largely to the relative abundance and reactivity of thiol groups. This interaction involves species with NO⁺ character, resulting from a one electron oxidation of NO, which strongly support the nitrosylation of protein thiols, with the formation of S-nitrosothiols (RS-NO) (239). Prominent amongst these is S-nitrosoglutathione (79), the product of the reaction of NO with the principal intracellular, non-enzymatic antioxidant, glutathione. RS-NO formation has been proposed as a means by which the potentially cytotoxic effects of NO and its reaction products, particularly peroxynitrite and other NO derived pro-oxidants, can be limited (80). Reaction of NO with protein thiol groups has been shown
to inhibit a wide range of enzymes (239), and to regulate a number of cell functions, including excitatory neurotransmission (144) and protein kinase C (PKC) signaling in cytokine-activated Mφ (85).

1.2.2.2 Reactive Oxygen Species in Acute Lung Injury

ROS, as listed above, represent potentially injurious cellular products. Generated in the course of normal metabolism, their toxicity is limited by intracellular defense mechanisms. When ROS production increases, however, these systems can become overwhelmed and tissue injury can occur.

Figure 2. Formation of reactive oxygen species.

In the course of normal aerobic metabolism in mammalian cells, oxygen (O₂) undergoes sequential, one electron reductions, with the formation of intermediate ROS. This is mediated in large part by the mitochondrial enzyme cytochrome oxidase, which accounts for the metabolism of approximately 98% of consumed O₂ (250) to two molecules of water (H₂O). In this
setting, intermediate ROS remain bound to the enzyme and are not released (121). A small proportion of O₂ consumption, however, leads to the formation and release of O₂⁻ and hydrogen peroxide (H₂O₂) (71). Although these particular ROS exhibit only limited reactivity in aqueous solutions (250), further reactions can lead to the formation of far more unstable and potentially injurious products (94), including OH⁻, and, as outlined above, peroxynitrite (Figure 2). To prevent this, intracellular enzymatic and non-enzymatic antioxidants act to maintain low levels of O₂⁻ and H₂O₂ (250) (Figure 3), as described further below. Various pathophysiological states, however, are associated with an increased production of ROS that exceeds the capacity of such defense mechanisms, with resultant tissue injury.

**Figure 3** Endogenous Antioxidants

$$2O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

$$2GSH + H_2O_2 \xrightarrow{\text{glutathione peroxidase}} GSSG + 2H_2O$$

$$2GSH \xrightarrow{\text{glutathione reductase}} 2GSH + 2NADH + 2NADP^+$$

Increasing the oxygen tension in the cellular environment is one such situation. This is most commonly an iatrogenic injury arising from the need to provide supplemental inspired oxygen in a critical care setting. Exposure of patients to 100% O₂ can result in
typical acute lung injury, characterized by alveolar flooding with protein-rich fluid, over a period of days (68). Physiological changes can be documented much earlier. Evidence for increased lung permeability is seen in humans after only 17 hours of exposure to 100% O₂ (54). Such exposure leads to large increases in microsomal and, particularly, mitochondrial H₂O₂ production in lung tissue (259).

The metabolism of drugs and chemicals can also result in increased ROS production and resultant tissue injury in a process termed "redox cycling" (250). Paraquat, for instance, is a herbicide that causes lung injury in humans (94) through the release O₂⁻, a process that may involve the generation of peroxynitrite, as it is attenuated by NOS inhibition (19). In addition, cigarette smoke also imposes oxidative stress by a number of mechanisms, including the redox cycling of hydroquinones to form O₂⁻ and H₂O₂ (204). Other enzymatic mechanisms exist for the production of excessive ROS under certain circumstances. In the setting of ischemia-reperfusion injury, xanthine oxidase catalyzes the conversion of hypoxanthine to xanthine at the time of reperfusion, with the release of O₂⁻ and, subsequently, H₂O₂ (250).

As indicated above, activated phagocytes release large quantities of ROS in the setting of inflammation. A wide range of stimuli can cause this release, which results from activation of a membrane-associated reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme (228). Compelling evidence also exists for the importance of oxidative stress, which is likely primarily phagocyte-derived, in patients with ARDS (see below).

When the production of ROS overwhelms intracellular defense mechanisms,
targets for oxidant-mediated damage include DNA, proteins and membrane lipids (94). DNA single-strand breaks can result from exposure to oxidant stress (113). ROS can oxidize protein sulfhydryl groups (238), with potential loss of function (224). Lipid peroxidation can also occur as a result of a chain reaction initiated by the formation of a lipid alkyl radical (L·) as a result of oxidant stress. This then undergoes subsequent oxidation to form lipid peroxyl (LOO·) and alkoxy (LO·) radicals which then perpetuate the process by acting as ROS (250) (figure 4).

Antioxidants As indicated, mechanisms exist to limit such potentially devastating effects of oxidant stress. Enzymatic antioxidants include superoxide dismutases, catalases and glutathione peroxidases (Figure 3). Superoxide dismutases (SOD) catalyze the conversion of O₂⁻ to H₂O₂ (250). These are metalloproteins, with copper, zinc (Cu,Zn)-SOD found largely in the cytosol, and manganese (Mn)-SOD located primarily in the mitochondria. SOD activity exists outside cells, also, in the form of extracellular (EC)-SOD. H₂O₂ is then converted to H₂O and O₂ by catalase or glutathione peroxidase (250).

Non-enzymatic antioxidants include the lipid-soluble compound α-tocopherol,
uric acid and the cytosolic non-protein thiol, glutathione (GSH). These act as a second line of defense, to limit the toxicity of ROS that elude enzymatic reduction. α-tocopherol is localized to cellular and subcellular membranes and acts largely to limit ROS-induced lipid peroxidation (250) through the donation of a hydrogen atom to LOO• and LO• compounds. Uric acid also represents a non-enzymatic antioxidant present at biologically significant concentrations in alveolar lining fluid (14,197).

GSH is generally the most abundant intracellular thiol, at concentrations of approximately 0.5-10 mM (224), and represents the principal non-enzymatic antioxidant. It is synthesized in all mammalian cells and its metabolic pathway is depicted in figure 5. The regulatory enzymatic step in this ATP-dependent process is catalyzed by γ-glutamylcysteine synthetase, which is under feedback control by GSH (55). The necessary amino acid substrates, cysteine, glutamate and glycine, are provided by transmembrane transport of amino acids and dipeptides, and the

Figure 5 Glutathione Synthetic Pathway

\[
\text{GLUTAMATE} \quad \text{CYSTEINE} \quad \text{GLYCINE} \\
\begin{align*}
\text{CH}_2\text{SH} & \quad \text{H}_2\text{NCHCOOH} \\
\text{ATP} & \quad \text{ADP} + P_i \\
\gamma\text{-glutamylcysteine synthetase} & \quad \gamma\text{-GLU-CYS} \\
\text{GSH synthetase} & \quad \text{GLYCYNE} \\
\text{H}_2\text{NCH}_2\text{COOH} & \quad \text{Gluathione (GSH)}
\end{align*}
\]
availability of cysteine is likely the true rate limiting step for GSH synthesis (224). Cysteine also represents the thiol containing component of GSH (figure 5).

GSH exerts antioxidant effects through several mechanisms, including the direct scavenging of ROS (250). As indicated above, it does play a role in the enzymatic reduction of H$_2$O$_2$, mediated by glutathione peroxidase (55) (Figure 3). This results in the formation of oxidized glutathione (GSSG), which is reduced by glutathione reductase in an NADPH-dependent process. Although catalase also mediates the reduction of H$_2$O$_2$, the majority of this ROS is catabolized by the glutathione peroxidase system in many cells (224). In addition, this system accounts for a similar reduction of lipid peroxides (55), in addition to $\alpha$-tocopherol.

GSH can also act to maintain and protect protein sulfhydryl groups, and, therefore, preserve protein function (224). GSH is capable of reacting with the disulfide and sulfoxide products of protein-ROS interaction and, consequently, reverse some of these injurious reactions (146). Indeed, the intracellular thiol/disulfide state of proteins is in equilibrium with the GSH/GSSG ratio (260). Similarly, oxidant-induced damage to DNA may be attenuated by GSH (224).

The maintenance of intracellular GSH levels depends on its rate of synthesis, related to enzymatic activity and substrate availability, and rates of utilization and salvage. Severe depletion of GSH can increase susceptibility to oxidant stress and disrupt basic cell functions (260). Glutathione reductase is usually effective in reducing GSSG, but can become overwhelmed in the face of rapid oxidation of GSH (55). In keeping with this, sepsis-associated ARDS has been shown to result in a depletion of
alveolar fluid total glutathione (194), as compared to the high levels seen in the alveolar epithelial lining fluid of normal individuals (38). This is consistent with additional evidence for the importance of oxidant stress in ARDS. Analysis of BAL fluid from patients with ARDS demonstrated evidence of protein oxidation (47), while increased H₂O₂ levels are seen in the expired breath of patients with ALI (9,235).

**Exogenous antioxidants.** In view of the importance of endogenous antioxidant defenses, as well as evidence for their depletion due to oxidant stress associated with a number of pathophysiologic situations, including ALI, numerous attempts have been made to devise therapies designed to limit ROS-induced injury. This has generally consisted of the administration of agents designed to augment endogenous antioxidants. Such strategies have included the use of exogenous SOD, catalase and α-tocopherol (250). The only antioxidant therapy accepted for widespread clinical use, however, is one involving the use N-acetylcysteine (NAC).

NAC, as its name would suggest, is an N-acetylated form of cysteine which, when deacetylated, can act as a potential source of the critical GSH precursor, cysteine. Direct administration of GSH has been largely ineffective in increasing intracellular stores due to the generally poor transmembrane transport of intact GSH (72,250), although some cell types are capable of GSH uptake (224). NAC has been used extensively in the treatment of acetaminophen/paracetamol poisoning (72), which is characterized by an oxidant-mediated hepatic injury resulting from a metabolic product of acetaminophen. NAC has also been used as a mucolytic agent in the
treatment of chronic obstructive pulmonary disease (COPD) (208,284). A number of in vivo experimental studies have also suggested a protective effect of NAC in acute lung injury. These include endotoxin injection in porcine and sheep models of acute lung injury (20,165) and microembolism- (269) and immune complex-induced (214) lung injury in rats. NAC was shown to prevent the leak of protein-rich fluid into the lung, as well as lung PMN influx and histologic changes, resulting from intratracheal instillation of IL-1 (136). This was associated with a decrease in expired H₂O₂ levels as compared to rats treated with IL-1 alone. Similar results have been shown in animals exposed to hyperoxia, including dogs (266), preterm guinea pigs (133) and rats (215). NAC was also shown to be effective in preventing DLEC cytotoxicity resulting from in vitro exposure to 95% O₂ (46). Given these and other promising data regarding the use of NAC in lung injury, as well as the extensive previous experience with the use of NAC clinically, trials in humans were undertaken. The benefit of NAC treatment in patients with ALI, however, remains unclear. While a recent prospective, randomized study did show evidence of improvement in lung function, no decrease in overall mortality was demonstrated (248). An earlier study involving patients with more severe lung injury failed to show any benefit in terms of survival or physiologic parameters (122). This likely reflects, at least in part, the difficulty in achieving adequate tissue and intracellular concentrations with any exogenously administered antioxidant.

The mechanism by which NAC acts remains somewhat controversial. As a typical thiol compound, it is capable of direct reaction with ROS. NAC has been shown in vitro to be a powerful scavenger of OH⁻ as well as hypochlorous acid, a ROS
produced by PMN-derived myeloperoxidase (3). As mentioned above, NAC is also capable of providing a source of cysteine and, consequently, stimulating the synthesis of GSH. This is the accepted means by which NAC exerts its antioxidant effect in the treatment of acetaminophen poisoning (72,135,167). NAC prevented the decrease in tissue GSH resulting from exposure to cigarette smoke in an isolated, perfused rat lung model, through stimulation of GSH synthesis (166). Christie and colleagues demonstrated that DLEC cytotoxicity resulting from hyperoxia was associated with a profound decrease in intracellular non-protein thiols. Not surprisingly, these cells were rendered more susceptible to this form of injury by GSH depletion with buthionine sulfoxamine (BSO), a γ-glutamylcysteine synthetase inhibitor (46). As indicated above, NAC treatment offered protection against this O₂-mediated cytotoxicity, and was associated with a prevention of the loss of non-protein thiols. Increases in intracellular glutathione levels have also been shown in Chinese hamster ovary cells as a result of NAC treatment (118). This was dependent on the presence of oxidized cysteine, or cystine, in the culture medium, and was associated with an increase in the uptake of radiolabelled cystine. Cell uptake of cystine is less efficient than that of cysteine, and it was proposed that NAC acted to reduce cystine to cysteine through a mixed disulfide reaction, facilitating the transmembrane transport of this GSH precursor. Phelps and colleagues (201) also demonstrated a similar cystine-dependent increase in GSH levels in cultured bovine pulmonary artery endothelial cells in response to NAC that was associated with increased cysteine transport. Higher concentrations of NAC, however, were able to reproduce this effect in the absence of cystine, indicating that NAC itself
might be transported into cells and thus directly support GSH synthesis. Cotgreave and colleagues (49) suggested the presence of an N-deacetylase on human endothelial cells that facilitates such transport at adequate NAC concentrations. This same study, however, showed NAC to be a relatively poor GSH precursor, increasing intracellular levels only after prior GSH depletion, and after a significant time lag. The authors went so far as to state that any cytoprotective, antioxidant effect of NAC likely depends on its scavenging ability as opposed to any significant effect on cellular glutathione.

The data regarding the *in vivo* action of NAC would similarly suggest some difficulty in modulating cellular GSH levels without prior depletion. Administered enterally, NAC is subject to extensive first pass metabolism in the liver, resulting in an oral bioavailability of only 10% (72). Intravenous NAC is rapidly oxidized in plasma to form disulfide species, and its half-life is between 2-6 hours (72). In preterm guinea pigs exposed to hyperoxia, protection from lung injury by intravenous NAC was not associated with an increase in whole lung GSH levels (133). Oral NAC administration did result in increased lung GSH levels in rats pretreated with diethylmaleate, an agent which depletes cellular GSH stores (214). Oral NAC administration to humans with no lung injury over a 5 day period resulted in increases in plasma cysteine levels, and an early, short-lived increase in BAL GSH concentration that had returned to baseline levels by 16 hours (50). A subsequent study in patients undergoing lung resection for tumours showed no increase in lung tissue GSH levels from an even higher oral dose of NAC (28). Burgunder and colleagues (33) demonstrated an increase in plasma GSH levels in human volunteers only after the administration of 2g acetaminophen. Patients
with sepsis-induced ARDS showed significant decreases in plasma and red blood cell
cysteine and glutathione levels which were ameliorated by intravenous NAC, with levels
rising within 24 hours and peaking at 96 hours (20).

1.3 Fluid and Solute Movement Across the Alveolar-Capillary Membrane

The endothelial and epithelial barriers are semi-permeable membranes, and
transmembrane fluid movement is usually evaluated using the Starling equation (243):

\[ Q_f = K_f[(\Delta P_{\text{hydro}}) - \sigma(\Delta P_{\text{osm}})] \]

\( Q_f = \) net transmembrane flow

\( K_f = \) membrane fluid conductance coefficient

\( \sigma = \) solute reflection coefficient

\( \Delta P_{\text{hydro}} = \) transmembrane hydrostatic pressure gradient

\( \Delta P_{\text{osm}} = \) transmembrane osmotic pressure gradient

\( K_f \) is the product of the barrier's fluid permeability per unit surface area and the total
surface area available. \( \Delta P_{\text{hydro}} \) is the result of hydrostatic pressure differences across
the membrane, and, in the case of the capillary barrier, between blood vessels and the
interstitium. Similarly, \( \Delta P_{\text{osm}} \) reflects relative osmotically active solute concentrations in
these areas while the solute reflection coefficient is a measure of the permeability of the
barrier to a given solute. This latter factor determines if a particular solute is, in fact,
osmotically active and capable of generating transmembrane osmotic pressure gradients. Specifically, the less permeable a barrier is to a given solute, the greater the $\sigma$ and the osmotic pressure exerted by that solute. As explained below, the endothelial and epithelial layers have significantly different reflection coefficients for solutes.

All factors which regulate normal fluid movement and the pathological alveolar flooding seen in ARDS can be considered within this framework.

1.3.1 Capillary Endothelial Barrier

Fluid accumulation in the alveolus begins with its movement out of the capillaries and into the interstitial space. The capillary is lined by endothelial cells with intervening tight junctions (217), the nature of which result in intercellular gaps equivalent to approximately 40 Å. These "tight" junctions normally allow the passage of fluid and plasma macromolecules into the interstitial space (251), to be cleared by the lymphatic system (244). Given the size of the intercellular gaps between endothelial cells, plasma proteins, but not smaller solutes, are the pertinent osmotically active species. Since the plasma has, relative to the interstitium, a greater protein concentration, the net osmotic force favours fluid movement into the vascular space. There is, however, a greater net transvascular hydrostatic pressure gradient, resulting from the positive intravascular and negative interstitial pressure. Taken together, this results in a net fluid efflux from the pulmonary microcirculation, even in the normal lung. In ALI, injury to the lung endothelium results in an increase in permeability, and this, combined with frequently observed pulmonary hypertension (263), promotes even greater movement of proteins.
and fluid into the interstitium. When the rate of fluid accumulation exceeds the
clearance capability of the lymphatic system, alveolar flooding can occur.

1.3.2 Alveolar Epithelial Barrier

Relative to the endothelial barrier, the alveolar epithelium forms an even more
restrictive barrier to fluid and solute movement. In addition, distinctive cellular
properties contribute significantly to the maintenance and, in the setting of alveolar
flooding, to the recovery of a fluid-free alveolar space.

The alveolar epithelium also possesses tight junctions (217), the nature of which
contribute to the highly resistant alveolar epithelial barrier. The effective molecular
radius of the alveolar inter-epithelial spaces is equivalent to approximately 4 Å, which
even restricts the movement of small ions such as sodium (Na⁺) and chloride (Cl⁻).
Ions, therefore, are the principal osmotically active species with respect to the normal
alveolar epithelial barrier, given their abundance in the interstitial space (approximately
300 mOsm/L) versus proteins, which represent approximately 1.5 mOsm/L. The
resulting osmotic pressure gradient opposes movement of fluid from interstitium to
alveolar space.

Alveoli are lined by two types of epithelial cells. Although AT1 epithelial cells
cover more than 95% of the alveolar surface area, they comprise only approximately
1/3 of the total alveolar epithelial cell number (93), and the more abundant ATII
epithelial cells represent the most important cellular contributor to alveolar fluid balance
by virtue of their unique properties.
ATII epithelial cells secrete surfactant, which is a lipid-rich substance that reduces surface tension at the air-liquid interface in the alveolus. Inhibition of surfactant function is seen in ARDS (89,95), largely mediated by plasma proteins (138) in the alveolar space, the most potent of which are fibrin(ogen) and fibrin degradation products (189,221). The resultant rise in surface tension leads to increased leakage of fluid from the surrounding capillaries (210).

ATII epithelial cells also replicate following epithelial injury, acting as progenitor cells for ATI epithelial cells (1) at a time when alveolar epithelial barrier function is compromised and integrity of the alveolar membrane is essential.

Finally, as discussed below, the ATII epithelial cell's ability to actively transport Na⁺ from apical (alveolar) to basolateral (interstitial) compartments (44) promotes fluid reabsorption from the alveolar space. Increasing evidence exists for the importance of this process in alveolar fluid clearance in general, and its derangement in ALI.

1.3.3 Alveolar Epithelial Na⁺ and Fluid Transport

The ability of alveolar epithelium to transport Na⁺ has been shown in numerous in vivo and in vitro models. Evidence is clear that this process is vital in the clearance of fetal lung fluid at the time of birth, and there is considerable data indicating its importance in the clearance of edema fluid in the post-natal lung.

ATII epithelial cells are clearly integral parts of normal lung function as well as the response and recovery to epithelial injury. Successful isolation and primary culture of adult (57) and fetal (203) ATII epithelial cells has led to a better understanding of the
basic mechanisms of alveolar epithelial ion transport. The isolation and primary culture of ATI epithelial cells has been largely unsuccessful and their role in Na⁺ absorption is unknown (183). Interestingly, with increasing time in culture, ATII epithelial cells demonstrate phenotypic characteristics consistent with ATI epithelial cells, including a flattened, squamous-like appearance (43) and immunoreactivity to monoclonal antibodies directed against ATI epithelial cells (58).

Early evidence supporting Na⁺ transport by ATII epithelial cells came from the observation that fluid-filled domes were formed beneath cultured monolayers (83,150), and that this process could be blocked by the drugs amiloride and ouabain. Amiloride is a potassium-sparing diuretic that blocks epithelial Na⁺ channels while ouabain is a specific inhibitor of the sodium-potassium- (Na⁺-K⁺)-ATPase, which is present on the basolateral surface of ATII epithelial cells (218). Na⁺ absorption has been shown to be an active process, requiring ATP, which could be augmented by agents that increased the levels of intracellular cAMP (52,82), including β₂-agonists. Active Na⁺ transport has also been observed in isolated, perfused rat lung, in which the appearance of intra-alveolar ²²Na⁺ tracer in the vascular perfusate was decreased by amiloride and ouabain and increased by a β₂-agonist (84). These and other data indicate that adult and fetal ATII epithelial cells transport Na⁺ through an active process, the rate limiting step of which is the movement of Na⁺ across the apical membrane via amiloride-sensitive Na⁺ channels (figure 6).
Several investigators have studied *in vivo* alveolar fluid clearance in a wide variety of species, including sheep (23), dogs (22), rabbits (230) and rats (120). All of these studies showed active, amiloride-sensitive fluid clearance within four hours of instillation of protein-rich serum into the lung. This has been directly assessed by measurement of lung water content and indirectly inferred from changes in fluid protein concentration, taking advantage of the relative impermeability of the alveolar epithelial barrier to protein (105). With the exception of rabbits, all species showed an increase in clearance in response to a β-adrenergic agonist which could be blocked by pre-treatment with amiloride. The process was found to be inhibited by ouabain in rats.
(120) and sheep (213). Recently, the model was extended to a resected human lung, with similar results (212). The active nature of this process was reflected by its impairment under hypothermic conditions (8°C).

Studies in newborn animals have demonstrated the importance of active Na⁺ transport in the clearance of fetal lung fluid at the time of birth, a process necessary for the adaptation to air breathing life. In newborn guinea pigs, pharmacological inhibition of Na⁺ transport with amiloride, or its analogues which are more specific for Na⁺ channels, resulted in an impairment of this liquid clearance with associated respiratory distress (185, 186). Similar severe pathophysiologic consequences resulted from the inactivation of the gene coding for a specific epithelial Na⁺ channel (ENaC) expressed in the lung (see below) (109).

More detailed information regarding lung epithelial ion transport came from studies using the short-circuit current (Isc) technique, developed originally by Ussing and Zerahn (262). Using what has come to be known as the Ussing chamber, the Isc technique allows the measurement of the bioelectric properties of epithelial monolayers, reflecting the function of ion conducting pathways, including Na⁺ channels. Epithelial cells are grown to confluence on permeable supports and mounted in chambers bathed on both sides with physiological medium, continuously bubbled with 95% air / 5% CO₂ and maintained at 37°C and pH 7.4. This configuration permits access to both sides of the monolayer, allowing study of the apical and basolateral components of the monolayer's bioelectric properties. Potential difference (PD) is measured directly and reflects the net vectorial ion transport and electrical resistance.
across the monolayer. The amount of externally applied current necessary to reduce the PD to zero represents the short circuit current ($I_{sc}$), an actual measure of the net ion movement. Transepithelial resistance ($R$) is determined by Ohm's law ($PD = I_{sc} \times R$), using the observed changes in PD resulting from known current pulses. $R$ is a sensitive measure of the functional integrity of the interepithelial tight junctions. Using this technique, then, the ion transport and barrier properties of cultured epithelial monolayers can be studied, as can the effects of various culture conditions and pharmacological interventions.

Using confluent monolayers of adult rat AT II epithelial cells in primary culture, Cheek and colleagues showed an apical to basal net transport of $Na^+$ which was equivalent to $4.37 \pm 0.24$ μA/cm$^2$ (44). The demonstrated $I_{sc}$ decreased by 80% with the apical administration of amiloride, and by 95% with the basolateral addition of ouabain. When ouabain was added to the apical surface, no effect was seen. Similarly, a much smaller and slower effect was observed when amiloride was added to the basolateral monolayer surface. In contrast, the addition of the $\beta_2$-agonist terbutaline resulted in an approximately 140% increase in $I_{sc}$, regardless of the side to which it was added. Net apical to basolateral $Na^+$ transport was confirmed with $^{22}Na^+$ flux measurements. Further studies in adult ATII epithelial cells have supported these findings and shown that substitution of $Na^+$ with an impermeant cation in the apical bath quickly reduced $I_{sc}$ to zero (151). Neither substitution of $Cl^-$ nor the addition of the $Cl^-$ transport inhibitor furosemide had any effect on monolayer bioelectric properties (151).
Extensive studies have also been conducted using fetal distal lung epithelial cells (DLEC), which are predominantly ATII epithelial cells, in primary culture. O'Brodovich and colleagues demonstrated, somewhat surprisingly, that DLEC isolated from 18- to 20-day gestational age (GA) fetal rats actively transport Na\(^+\) at rates similar to adult ATII epithelial cells in primary culture (185, 188). No evidence of Cl\(^-\) secretion by these cells was demonstrated. Baseline \(I_{sc}\) was significantly reduced by amiloride (by a factor of 66%) or its analogue, benzamil, which exerts a more specific inhibitory effect on the Na\(^+\) channel. Another amiloride analogue, more active against the Na\(^+\)-H\(^+\) antiporter, dimethylamiloride, and phloridzin, which inhibits the Na\(^+\)-glucose cotransporter, had no effect on \(I_{sc}\). Similarly, a number of Cl\(^-\) channel inhibitors did not change baseline bioelectric properties. Terbutaline resulted in a two-fold increase in \(I_{sc}\), and this effect was blocked by amiloride. Addition of ouabain to the basolateral surface again reduced \(I_{sc}\) to almost zero. A similar study confirmed that the resting and terbutaline-stimulated \(I_{sc}\) was dependent on Na\(^+\) in the apical bathing medium (185).

Although the above studies demonstrated that majority of \(I_{sc}\) was amiloride-sensitive, some net ion movement persists after amiloride treatment. The exact nature of this ion flux is not known. Specifically, it is unclear if this is the result of the apical to basolateral absorption of Na\(^+\) along amiloride-insensitive pathways, or active Cl\(^-\) secretion in the opposite direction. Evidence from studies with bovine tracheal epithelium would suggest that it is due to residual, amiloride-insensitive Na\(^+\) transport (134). Amiloride treatment blocked only 58% of the \(^{22}\)Na\(^+\) active transport, a direct demonstration of significant amiloride-insensitive Na\(^+\) transport. Although studies in
mature fetal DLEC (188) failed to show any effect of Cl\(^-\) channel inhibitors on \(I_{sc}\) (see above), DLEC from immature, day 18 GA fetal rats do demonstrate some Cl\(^-\) secretion (12).

1.4 Sepsis-Related Injury to the Alveolar-Capillary Membrane

1.4.1 Endothelial Injury

The susceptibility of endothelium to inflammatory injury has been extensively studied, and will not be described in depth here. Leakage of fluid and protein from the lung vasculature, as measured by experimental techniques such as increases in lung lymph flow (245) and the accumulation of protein tracer in the extravascular space (272), has been demonstrated in a number of in vivo models of ALI, particularly infectious ones. Infusion of endotoxin, live bacteria or other suspected mediators of sepsis (169) all resulted in increased vascular permeability.

1.4.2 Epithelial Injury

For there to be alveolar flooding, the normally tight barrier to fluid and solute movement formed by the distal airway and alveolar epithelium must be breached (157). Numerous studies have demonstrated that the alveolar epithelial barrier is much more resistant to sepsis-related injury than the capillary endothelium. Intravenous infusion of *Escherichia coli* endotoxin in sheep was shown to significantly increase lung lymph flow and interstitial, but not intra-alveolar, accumulation of a vascular protein tracer (273).
Alveolar total protein concentration was unchanged over a period of 24 hours, again indicating no change in the protein permeability characteristics of the alveolar epithelium despite increased protein flux from the vascular space. Even more notable is the fact that epithelial fluid transport out of the alveoli was maintained, as reflected by an increase in the protein concentration of instilled, protein-rich fluid. This ability was preserved even after intra-alveolar instillation of endotoxin, despite a significant accumulation of PMN within the alveoli. Similar findings were confirmed in rats (202) infused with live *Pseudomonas aeruginosa*. In this study, the rate of alveolar fluid clearance was, in fact, doubled in bacteremic rats, associated with a significant rise in plasma epinephrine levels. This latter effect could be blocked both by amiloride and by β-adrenergic receptor antagonists, indicating a receptor-mediated increase in Na⁺ channel activity, consistent with findings in normal lungs. This apparent *in vivo* stimulation of epithelial Na⁺ transport and fluid clearance in response to an inflammatory insult is particularly compelling evidence for the physiological importance of this highly resistant barrier and the role of active Na⁺ transport as a vital component of it.

Several experimental insults are, however, capable of compromising alveolar epithelial barrier function. The intra-alveolar instillation of large numbers of *P. aeruginosa* in sheep (273) and rabbits (274) resulted in an increased alveolar protein permeability and decreased fluid clearance. Similar findings were shown with intra-alveolar oleic acid (273) and prolonged hyperoxia (76). Important to note, however, is the fact that, despite significant functional and morphological injury, the alveolar
epithelium was able to maintain or recover some capacity to clear fluid from the airspace.

Epithelial integrity and active alveolar fluid clearance has been studied in patients and found to be important for their survival. Rubin and colleagues studied patients with demonstrated septic shock and showed that all had clinical and biochemical evidence of endothelial injury but only 30% developed alveolar flooding (211). Using changes in serial bronchoalveolar lavage (BAL) fluid protein concentrations as an index, Matthay and colleagues demonstrated that the ability to progressively reabsorb airspace fluid correlated with survival in 16 intubated patients with ALI (158). Among 9 such patients who demonstrated a progressive increase in BAL fluid protein concentration, reflecting fluid reabsorption, the mortality rate was 22%. In the remaining 7 patients, the death rate was 71%. The concentration of BAL fluid protein rose to levels higher than those in plasma, providing evidence in the first group of patients for an active process of fluid reabsorption. This would seem to indicate that the ability of the residual alveolar epithelial barrier to effectively carry on the process of alveolar fluid clearance is related to ultimate survival from ALI. This might reflect a less profound, or perhaps less generalized, initial injury with a quantitatively more intact barrier, or compensatory mechanisms in residual alveolar epithelial cells. As suggested in animal studies, this may well include enhanced epithelial Na⁺ transport. Of note, the mean alveolar fluid clearance rate estimated among patients who concentrated their BAL fluid protein (18 ± 15% / h) was relatively fast compared to that observed in other
species (4 ± 2% / h in dogs, 8 ± 3% / h in sheep, and 15 ± 4% / h in rabbits, data expressed as mean ± SD).

Recent in vitro data from our laboratory may provide one, at least partial explanation as to why some of the septic patients in Matthay’s study (158) were unable to absorb airspace fluid. Studying fetal DLEC in primary culture, Compeau and colleagues showed that a soluble factor released from endotoxin-stimulated alveolar macrophages (AM) resulted in an impairment of amiloride-sensitive Na⁺ transport through a mechanism dependent on the presence of L-arginine, the specific substrate for nitric oxide (NO) production (see below), which was partially prevented by treatment with N⁵-monomethyl-L-arginine (NMMA), a competitive inhibitor of the enzyme responsible for NO synthesis (48). An important role for NO in this process was further suggested by experiments using S-nitroso-N-acetylpenicillamine (SNAP), a direct, non-enzymatic NO donor, which mimicked the effect on DLEC Na⁺ transport (56). A decrease in the epithelial membrane density of a specific functional Na⁺ channel, the non-selective cation channel (see below), resulted both from exposure to LPS-stimulated AM (48) and SNAP (56). This in vitro model of ALI appears, however, to affect Na⁺ channel function through different mechanisms. NMMA resulted in only partial reversal of the impairment of DLEC Na⁺ transport, indicating an NO-independent component of this process. In addition, cell-free supernatants from LPS-stimulated AM caused a similar impairment in DLEC Na⁺ transport, an effect which was unaltered by treatment of DLEC with NMMA (48).
Taken together, the data suggest that Na\(^+\) transport is an integral component of alveolar-capillary barrier function which may well be compromised in ALI, as suggested both by experimental models and patients with ARDS. Given this, an appreciation of the more basic mechanisms of epithelial ion transport, and specifically the properties of epithelial Na\(^+\) channels, is necessary to more fully understand this basic functional unit of the lung.

1.5 Epithelial Na\(^+\) Channels

As outlined above, the alveolar epithelium plays a vital role in the maintenance of alveolar fluid balance, both by forming a tight barrier to fluid entry into, and by mediating fluid transport out of, the airspace. The characteristics of this transport function, and specifically the pharmacological regulation of it, demonstrate a likely role for epithelial Na\(^+\) channels in ensuring an essentially fluid-free alveolar space, and, consequently, adequate gas exchange.

Much has been learned in the past decade regarding the molecular mechanisms of alveolar epithelial ion transport as the fundamental importance of this process has become increasingly recognized. A number of basic questions remain, however, arising in large part from the study of different cell populations, varied isolation and culture techniques and differing experimental models. Confusion exists particularly regarding the relationship between the various channels that have been functionally and structurally characterized, and the roles they play in the tissues in which they have
been identified. Important to this discussion is the relationship between the Na\(^+\) conducting channels that have been described in lung epithelium and those, perhaps better characterized channels in other epithelial cell types.

1.5.1 Na\(^+\) Channels in Non-Pulmonary Epithelia

A number of epithelia have as their principal function the transport of fluid and solute, including cells of the distal and collecting tubules of the kidney, distal colon, sweat glands and lungs. What the epithelial cells of these varied organs have in common are apically-located, Na\(^+\)-conducting channels (234), which are characteristically inhibited by amiloride (16). These represent the rate limiting step in a process of vectorial Na\(^+\) transport down a favourable electrochemical gradient maintained by basolaterally located Na\(^+\)-K\(^+\)-ATPases (Figure 6). These and other ion channels act to tightly control the amount and solute composition of luminal (apical) fluid in their respective organs. Functional characterization of these ion conducting pathways has principally involved the \(I_{sc}\) method, described above, and the patch-clamp technique (96).

Unlike the Ussing chamber, which assesses epithelial ion channel activity indirectly by measuring the bioelectric properties of epithelial monolayers, patch-clamp techniques, using fine pipettes as microelectrodes, allow measurement of single channel properties, including ion selectivity and conductance. Mean open and closed times, referred to as gating kinetics, can be determined by measuring ion fluxes over time. Open probability (\(P_o\)) refers to the likelihood of a channel being in the open state.
at a given time. In addition, patch-clamping allows the close examination of channel regulatory pathways since small portions of membrane containing individual channels can be excised, permitting complete control of the resulting microenvironment.

As a result of studies using these and other techniques, a number of Na⁺ channels with apparently distinct properties have been functionally characterized in various non-pulmonary "tight" epithelia. These can be categorized based on their ion selectivity, and further differentiated by their conductance, and gating properties (Table 6) (195). All are highly sensitive to amiloride. As can be seen, highly selective channels almost exclusively transport Na⁺ and have low single channel conductance. They exhibit slow gating kinetics, with relatively long open and closed times and are

<table>
<thead>
<tr>
<th>Na⁺ Channel</th>
<th>Pₙa/Pₖ</th>
<th>Conductance</th>
<th>t₀pₖ/tCl₀</th>
<th>Kᵢ (amiloride)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Highly selective (e.g. Rat CCT)</td>
<td>&gt; 10</td>
<td>4.9</td>
<td>3.4/3.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Mod. selective (e.g. A6 cells)**</td>
<td>3-4</td>
<td>7-10</td>
<td>0.04/0.1</td>
<td>0.9</td>
</tr>
<tr>
<td>NSC Channels (e.g. rat IMCD)</td>
<td>1</td>
<td>28</td>
<td>0.04/0.05</td>
<td>&lt; 0.1</td>
</tr>
</tbody>
</table>

Pₙa/Pₖ - Na⁺ to K⁺ selectivity ratio
Kᵢ - concentration for half-maximal inhibition
CCT - cortical collecting tubule
IMCD - inner medullary collecting duct
** when cultured on plastic

* adapted from (195)
regulated by a number of mechanisms including hormonal modulation by aldosterone and antidiuretic hormone (ADH), and by intracellular and extracellular ion concentrations.

Aldosterone increases apical Na⁺ permeability in renal and colonic epithelium, on a time scale of hours to days (195). There is an early response that may involve the direct activation or membrane insertion of existing channels and a later effect dependent on protein synthesis (77). This probably is not the result of de novo synthesis of the channel itself as these effects do not involve an increase in total channel protein, as estimated by amiloride binding or immunoprecipitation, and are susceptible to trypsin hydrolysis of existing membrane proteins (78). Evidence that aldosterone may, however, influence synthesis of channel regulatory proteins includes data from studies performed on rats maintained on a low salt diet, resulting in increased aldosterone levels (143). Colonic epithelium was analyzed in the Ussing chamber and a progressive rise in amiloride-sensitive \( I_{sc} \) was observed, paralleled by a similar rise in the mRNA level for a non-pore forming subunit of a specific epithelial Na⁺ channel, ENaC (see below). Messenger RNA for the putative pore-forming subunit was unchanged.

ADH mediates an increase in renal epithelial Na⁺ channel activity within minutes through activation of adenylate cyclase (78). Subsequent events are unclear but may involve channel recruitment from an intracellular pool and/or protein kinase A (PKA)-mediated protein phosphorylation.
Prevailing ion concentrations also modulate channel activity. Increased intracellular Na\(^+\) results in a negative feedback effect on channel activity (78), with a consequent decrease in Na\(^+\) permeability and prevention of excessive changes in cell volume. This may well involve an increase in intracellular calcium (Ca\(^{2+}\)) concentrations resulting in a protein kinase C (PKC)-mediated inhibition of Na\(^+\) channel activity. Similarly, Na\(^+\) permeability has been shown to bear an inverse relationship to extracellular (luminal) Na\(^+\) concentrations, also possibly involving Ca\(^{2+}\) and PKC interactions (234).

Moderately selective Na\(^+\) channels have also been identified and characterized. These cation selective channels are less selective for Na\(^+\), relative to K\(^+\), and exhibit higher single channel conductance and faster gating kinetics than the highly selective channels. Although described in a number of cell types, these channels have been studied most in A6 cells, an amphibian renal epithelial cell line, where they coexist with highly selective Na\(^+\) channels. Moderately selective Na\(^+\) channels predominate when cells are cultured on non-permeable supports and have not been identified in fresh tissue (78, 195). Given this and other evidence, Palmer has suggested that possibly the highly and moderately selective Na\(^+\) channels are in fact interconvertible, with their selectivity being a labile characteristic influenced by isolation and culture conditions (195).

A third group of channels, while still impermeant to anions, conduct Na\(^+\) and K\(^+\) equally and are therefore designated non-selective cation (NSC) channels. Exhibiting higher single channel conductance and relatively fast gating kinetics, these channels
have been found in cells cultured from rat inner medullary collecting ducts (IMCD) (142). Extensive studies with these cells demonstrated regulation of the NSC channel by cyclic GMP (cGMP)-mediated events (139-141). Atrial natriuretic peptide (ANP), a circulating hormone released from the atria in response to volume expansion, was shown to inhibit the channel by decreasing $P_0$. cGMP is a recognized intracellular second messenger for ANP in a variety of cells and membrane permeant analogues of cGMP were shown to similarly decrease NSC channel $P_0$ (141). These actions appear to involve both a direct, phosphorylation-independent process as well as activation of cGMP-dependent protein kinase (cGMP kinase) (140). The kinase-mediated events seem to occur through inactivation of guanine nucleotide binding proteins (G-proteins), a family of peptides composed of three subunits which couple plasma membrane receptors to a variety of enzymes and ion channels (139). Direct activation of G-proteins both blocked and reversed cGMP-kinase inhibition of the NSC channel (140). Given the regulation of the NSC channel by this important hormonal system, it seems likely that it plays an important role in vivo, at least in renal epithelial cells.

1.5.2 Functional Characterization of Na⁺ Channels in Lung Epithelium

In view of the vital importance of alveolar fluid balance and the large body of evidence for the ability of in vivo and cultured lung epithelium to transport Na⁺, it is not surprising that channels with similarly diverse functional characteristics have been identified in these cells. As indicated, their relationship to each other and to those channels described in other tissues is unclear.
The first ion channel to be identified in DLEC bore functional characteristics very similar to the NSC-type channel described above. Using fetal DLEC in primary culture, patch clamp studies revealed this completely cation selective channel with a conductance of $23 \pm 1.2 \, \text{pS}$, a $P_{Na}/P_K$ of 0.9, indicating no discrimination between these ions, a significant inhibition of $P_o$ by 1 $\mu$M amiloride, and a requirement for high cytosolic $Ca^{2+}$ levels for activation (193). It is now recognized to represent approximately 95% of identified channels in cultured fetal DLEC (193,255), and is also found in adult ATII epithelial cells (70). The fetal lung epithelial NSC channel in the excised patch clamp configuration has a very low $P_o$ at intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$) less than 1 $\mu$M (149), requiring a $[Ca^{2+}]_i$ of 1mM for maximal activation in otherwise unstimulated cells. Work in Marunaka’s laboratory has described how the NSC channel might be activated in vivo. The $\beta_2$-agonist terbutaline, known to stimulate $Na^+$ transport in fetal DLEC (188) and adult ATII epithelial cells (44), was shown to increase $[Ca^{2+}]_i$ to more than 1 $\mu$M in fetal epithelial cells (149). In addition, terbutaline acted to increase channel sensitivity to cytosolic $Ca^{2+}$ and to produce a dependency of the channel activity on cytosolic $Cl^-$ concentrations at physiologic $[Ca^{2+}]_i$ (254). These actions of terbutaline, therefore, provide possible explanations as to how the lung NSC channel is activated under physiologic conditions.

Other types of channels have been identified in fetal DLEC, albeit much less frequently. Voilley and colleagues (265) demonstrated the presence of a low conductance (4 pS), highly selective ($P_{Na}/P_K \geq 10$) $Na^+$ channel in the apical membranes of fetal DLEC in primary culture, very similar to the highly selective channel
in other epithelia described above. It was sensitive to amiloride ($K_i = 90nM$) and to the Na$^+$ channel specific amiloride analogues phenamil ($K_i = 19nM$) and benzamil ($K_i = 14nM$). By contrast, the channel was unaffected by 5-(N-ethyl-N-isopropyl) amiloride (EIPA), a derivative usually considered much less active against Na$^+$ channels (see below). Tohda and Marunaka have also identified a 12 pS, highly selective Na$^+$ channel in fetal DLEC, characterized by Ca$^{2+}$- independence and a somewhat lower amiloride sensitivity (255).

Not all Na$^+$-permeant channels are equally amiloride sensitive (152), and studies with fetal DLEC (152) and adult ATII (153) cells have suggested the presence of different pathways for Na$^+$ entry, differentiated based on their sensitivity to amiloride and its analogues. H-type channels are distinguished by a high amiloride and benzamil affinity ($K_i$ in the nanomolar range) and a low sensitivity to EIPA. L-type channels, in contrast, are equally sensitive to amiloride, benzamil and EIPA ($K_i$ in the μM range). $[\text{3}^\text{H}]$benzamil equilibrium binding studies in membrane vesicles from cultured fetal ATII epithelial cells revealed the presence of two binding sites with different dissociation constant ($K_d$) values (19 and 1525 nM, respectively), indicating both high and low affinity binding sites in these cells (152). In contrast, membrane vesicles from A6 cells, a renal epithelial cell line, demonstrated only a single, high affinity site ($K_d = 18nm$).

$^{22}\text{Na}^+$ uptake into fetal DLEC membrane vesicles was inhibited to a somewhat greater extent by EIPA than either amiloride or benzamil, suggesting a functional predominance of L-type channels in this setting (152). Similar results were shown in freshly isolated adult ATII epithelial cells, where an equal sensitivity of $^{22}\text{Na}^+$ flux into intact cells to
amiloride, benzamil and EIPA was demonstrated (154). Patch clamp analysis in these cells exclusively showed a channel with a $P_{Na}/P_K$ ranging from 3.16 to 2.34. Further studies have, in fact, failed to identify any H-type channels in freshly isolated and cultured adult ATII epithelial cells (98). These results would seem to indicate that the principal pathway for entry of Na$^+$ into ATII epithelial cells might be somewhat different from those identified in some renal epithelia (265).

In addition to Na$^+$ channels, several other potential Na$^+$ transport pathways exist. Na$^+$ exchangers, including the Na$^+/H^+$ antiport, and Na$^+$ cotransporter systems, such as the amino acid and glucose cotransporters, have been demonstrated in DLEC. These systems, however, do not seem to contribute significantly to electrogenic Na$^+$ transport in the lung (70,153,185). This may be related to their abundance or activation, but it is most likely a reflection of the relative ion conductance rates of these channels. Amiloride sensitive Na$^+$ channels transport Na$^+$ at a rate of 1 to 10 million ions per second, whereas the rate for the Na$^+/H^+$ antiport and other electrogenic exchangers is only approximately 2000 ions per second (151).

1.5.3 Regulation of Lung Epithelial Na$^+$ Channels

Differences between pulmonary and non-pulmonary Na$^+$ channel characteristics are emphasized when regulatory mechanisms for lung epithelial Na$^+$ transport are examined. Extensive work has been performed in this area utilizing the $I_{sc}$ technique with fetal DLEC or adult ATII epithelial cells.
CAMP The ability of agents that increase intracellular cAMP levels to stimulate lung epithelial Na\(^+\) and fluid transport was recognized early by the observation that β-agonists and membrane permeant cAMP analogues could increase dome formation and \(I_{sc}\) across adult ATII cell monolayers (83,150). This was consistent with the previously demonstrated role of β-agonists in the conversion of the fetal lung from a fluid-secreting to a fluid-absorbing organ. A similar increase in fluid absorption was demonstrated in intact adult lung (see above). The fact that these effects were blocked by amiloride strongly supported the concept that the stimulation of fluid absorption was occurring via membrane Na\(^+\) channels. The specific downstream events mediating cAMP-dependent stimulation of Na\(^+\) transport are unknown but may involve phosphorylation of channel or regulatory proteins. As cited above, β-agonists can stimulate fetal DLEC cell NSC channel activity by virtue of increasing intracellular Ca\(^{2+}\) concentrations and modulating channel sensitivity to Ca\(^{2+}\).

Given the role of ADH in modulating renal Na\(^+\) channel function through cAMP-mediated pathways, it might be expected to exert similar control in lung epithelium. While ADH can decrease the rate of lung fluid secretion by fetal goats and lambs (198), it occurs only when the lungs are overinflated (199). Specific experiments have not demonstrated an effect of ADH on Na\(^+\) transport in primary cultures of adult ATII epithelial cells (52) or fetal DLEC (187). In fetal cells, ADH failed to induce an increase in endogenous cAMP levels (187), suggesting that these cells lack the necessary membrane receptors.
Other Hormonal Factors Studies have also been done examining the other hormonal regulatory factors recognized to influence \( \text{Na}^+ \) channel function in other tissues, particularly renal epithelia. Given the apparent importance of the NSC channel in both the kidney and the lung, the role of ANP and intracellular cGMP in the regulation of lung epithelial \( \text{Na}^+ \) transport was investigated. ANP failed to increase \( \text{Na}^+ \) transport by the intact rat lung (190,191), consistent with the previous observation that bioelectric properties of adult ATII epithelial cells were unaffected by cGMP (52). Using fetal DLEC monolayers, O’Brodovich and colleagues similarly showed that neither ANP nor a membrane permeant cGMP analogue had any effect on baseline bioelectric properties in the Ussing chamber (187).

Similarly, aldosterone does not appear to significantly increase \( \text{Na}^+ \) transport in the lung, in contrast with its action in renal and colonic epithelium (187). When an effect is observed, it is mediated by aldosterone stimulation of glucocorticoid receptors (41,252).

It is not surprising that ion and fluid transport are differentially regulated in organs with functions as diverse as the kidney and the lung. These data, however, indicate not only a difference in hormonal regulation of \( \text{Na}^+ \) transport in these epithelia, which could be explained by membrane receptor profiles, but also in responses to intracellular signaling mechanisms. This may reflect, for instance, the presence of distinct channel regulatory proteins.
1.5.4 Biochemical Purification and Cloning of Epithelial Na\textsuperscript{+} Channels

1.5.4.1 Biochemical Purification of the Amiloride-Sensitive Na\textsuperscript{+} Channel

Initial attempts to identify and purify specific Na\textsuperscript{+} channel proteins relied on high affinity binding to radioactive or photoactive amiloride analogues. The best characterized epithelial amiloride binding protein was described by Benos and colleagues in renal epithelial cells (17). This approximately 700 kDa protein, when reconstituted into lipid vesicles and planar lipid bilayers, conferred amiloride-sensitive, Na\textsuperscript{+} selective channel activity. The protein resolved into six major polypeptides under reducing conditions (18), likely through disruption of disulfide bonds, ranging in molecular mass from 40 to 315 kDa. Evidence would suggest distinct roles for many of these subunits (78), with a 150 kDa protein representing the specific amiloride binding site. Three of the subunits are heavily glycosylated (234). A lower molecular weight protein (30-45 kDa) was subsequently found to be the \(\alpha\)-subunit of a G-protein (78), which is interesting in view of the important role of cGMP-mediated events in the regulation of the renal NSC channel. This subunit, as well as the 95 kDa subunit, are ADP-ribosylated by pertussis toxin, an agent known to inhibit G-protein activation. Regulatory phosphorylation events appear to involve different subunits specifically, with PKA activating channel function through interactions with a 315 kDa subunit, and both the 150 and the 55 kDa proteins acting as substrates for PKC-mediated channel
inhibition. The 71 and 95 kDa subunits may be involved in channel responses to aldosterone.

Antibodies raised against this putative channel protein localize to the apical membranes of a number of epithelia known to possess Na⁺ transporting capabilities, including adult ATII epithelial cells (234) and fetal DLEC (152). In western blot analysis of protein from freshly isolated adult ATII epithelial cells, the antibody recognized a 135 ± 10 kDa protein, whereas similar studies using protein from membrane vesicle preparations of cultured fetal DLEC (152) revealed binding to 150, 120, 90, 75 and 70 kDa proteins suggesting a different, oligomeric structure. Whether these represent distinct proteins or proteolytic fragments is unclear.

1.5.4.2 Cloning of an Epithelial Na⁺ Channel (ENaC)

Using a rat colon DNA library, Canessa and colleagues identified and sequenced a polypeptide which, when functionally expressed in Xenopus oocytes, conferred amiloride sensitive Na⁺ current (35). This protein was designated α-rENaC, for rat epithelial Na⁺ channel. Xenopus oocytes injected with the corresponding cRNA exhibited highly Na⁺ selective, amiloride sensitive current (Kᵢ = 0.1 μM), with slow single channel gating characteristics. Given that the magnitude of this current was smaller than that resulting from the similar injection of total cellular poly(A)⁺ mRNA, it was suspected that other subunits were important for the full expression of this channel's function. Additional subunits were subsequently identified and called β- and γ-rENaC (37). When all three subunits were expressed, more than a 100-fold potentiation of the
current resulting from α-rENaC injection alone was observed. Since neither β- nor γ-rENaC alone induced any current, it was concluded that α-rENaC represented the pore forming subunit while β- and γ-rENaC exert a regulatory role, important for maximal channel function. The conductance of the functional channel complex was 4.6 pS.

The size of the cDNA clones and resulting predicted size of the non-glycosylated proteins are listed in Table 7, along with the size of the RNA transcripts recognized by these clones on northern blots. They share 34-37% homology with each other and significant sequence similarities with mec-4, deg-1 and mec-10, a family of Caenorhabditis elegans genes involved in sensory touch transduction and, when mutated, the degeneration of neurons through excessive cell swelling (40,66). The distribution of rENaC is consistent with epithelia expressing highly selective Na⁺ channel activity. Specifically, all subunits were identified in rat colon and kidney cortex and medulla (37). Not surprisingly, α-, β-, and γ-ENaC are also found in both fetal

<table>
<thead>
<tr>
<th></th>
<th>α-rENaC</th>
<th>β-rENaC</th>
<th>γ-rENaC</th>
</tr>
</thead>
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<tr>
<td>O.R.F. (nucleotides)</td>
<td>3161</td>
<td>2534</td>
<td>3111</td>
</tr>
<tr>
<td>Protein size:</td>
<td></td>
<td></td>
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<tr>
<td>amino acids</td>
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<td>638</td>
<td>650</td>
</tr>
<tr>
<td>Predicted MW (kDa)</td>
<td>79</td>
<td>72</td>
<td>75</td>
</tr>
<tr>
<td>mRNA transcript (kb)</td>
<td>3.7</td>
<td>2.2</td>
<td>3.2</td>
</tr>
</tbody>
</table>

O.R.F. - open reading frame
kDa - kiloDaltons
kb - kilobases
and adult, rat and human lung (184,265). In fetal rat lung, rENaC expression appears
developmentally regulated, with α−transcripts appearing only in late GA fetal rats (184),
while β− and γ−subunits are significantly expressed only after birth (252). This
developmental expression can be hormonally influenced, with an earlier and greater
fetal α−rENaC expression with corticosteroid treatment of pregnant rats between 16
and 18 days GA (184,252). This is consistent with the in vivo observation that
combined corticosteroid and thyroid hormone treatment (10), or corticosteroid treatment
alone (11), can induce a Na⁺ and fluid reabsorptive capacity in immature fetal lamb
lungs. A glucocorticosteroid effect on α−rENaC expression (41), and amiloride-
sensitive I_{sc} (51), has also been demonstrated in cultured fetal DLEC. All three
subunits are strongly expressed in adult lung tissues from both rats (184,252) and
humans (117,265). The gene encoding α−hENaC (human) has been localized to
chromosome 12 (265). β- and γ-hENaC genes are tightly linked on chromosome 16
(227).

Consideration of the primary structure of the ENaC subunits has allowed
speculation regarding their conformation in epithelial cell membranes (35,37).
Hydropathy analysis revealed 2 hydrophobic domains and proposed structures include
2 putative transmembrane segments (36,37). A large predicted hydrophilic loop
contains conserved cysteine rich boxes and a number of potential N-linked
glycosylation sites, of which some have been shown to be glycosylated in living cells
(36). Both the amino- and carboxy-termini are predicted to be intracellular. Although
the oligomeric structure of the channel is unknown, Ismailov and colleagues have
recently proposed that functional ENaC comprises three identical conducting elements, presumably α-ENaC subunits, based on discrete subconductance levels observed in patch clamp studies of channels in the membranes of injected Xenopus oocytes or reconstituted in lipid bilayers (115).

As can be seen from the above discussion, a great deal of work has been conducted in an attempt to clarify the specific mechanisms involved in epithelial Na⁺ transport in the lung and other tissues. Several questions, however, remain to be resolved. Importantly, the contribution to net Na⁺ transport by the many channels that have been characterized, both functionally, biochemically and through molecular cloning techniques, is the subject of intense study. It seems increasingly certain, however, that ENaC represents a vital channel protein in the membranes of epithelial cells.

1.6 Experimental Hypothesis

Our laboratory has previously demonstrated that a soluble factor released from endotoxin-stimulated AM could impair fetal DLEC amiloride-sensitive Na⁺ transport through an L-arginine dependent mechanism (48,56). The importance of NO was confirmed with subsequent experiments showing that this effect could be reproduced by the non-enzymatic NO donor, SNAP (56). LPS-stimulated AM coculture was also shown to increase the expression of epithelial iNOS (56). It was also demonstrated, however, that impairment of DLEC Na⁺ transport could occur in different ways. Cell-
free supernatants from LPS-stimulated AM also resulted in a decrease in DLEC amiloride-sensitive $I_{sc}$, an effect which could not be prevented by treatment of DLEC monolayers with NMMA. The general aim of these experiments, therefore, was to clarify the specific mechanisms by which products from endotoxin-stimulated rat AM affected Na$^+$ transport in fetal rat DLEC.

It was hypothesized that the effect on DLEC Na$^+$ transport might be associated with modulation of gene expression for the specific Na$^+$ channel, ENaC. It was further postulated that the NO-mediated impairment of DLEC Na$^+$ transport might be the result of the generation of peroxynitrite in the coculture model. In addition, experiments were undertaken to investigate strategies designed to modulate the apparently varied effects of this specific in vitro model of ALI on DLEC Na$^+$ transport. The experiments described below, therefore, had the following specific aims:

1. To determine if exposure of DLEC to factor(s) released from endotoxin-stimulated AM directly or indirectly resulted in altered $\alpha$-, $\beta$-, and $\gamma$-ENaC mRNA levels.

2. To examine strategies whereby this decrease in rENaC gene expression could be prevented.

3. To detect the action of peroxynitrite in the coculture system, as reflected by the accumulation of nitrotyrosine residues on the membranes of DLEC.
4. To determine if NAC, an agent that has been used clinically in the treatment of patients with ARDS, could modulate the impairment of DLEC Na⁺ transport resulting from exposure to the cell-free supernatant of LPS-stimulated rat AM.
MATERIALS, METHODS AND PROTOCOLS

2.1 Materials and Solutions

Tissue culture media and additives including Hanks’ balanced solution (HBSS with and without Ca$^{2+}$ and Mg$^{2+}$), fetal bovine serum (FBS), Dulbecco’s phosphate-buffered saline (PBS), Eagle’s minimal essential media (MEM) and MEM selectamine kits were all obtained from Gibco Laboratories (Grand Island, NY). Collagenase and deoxyribonuclease for epithelial cell harvest were purchased from Worthington Biochem (Freehold, NJ). Trypsin and penicillin/streptomycin were obtained from Gibco Laboratories. Endotoxin (Escherichia coli 0111:B4) was from Difco Laboratories (Detroit, MI). NMMA was purchased from Calbiochem Behring (LaJolla, CA). $[^{14}\text{C}]$adenine used in epithelial cytotoxicity assays ($[^{8-^{14}}\text{C}]$adenine hydrochloride, 58.5 mCi/ml) was obtained from Du Pont (Boston, MA). Amiloride, bumetanide, flufenamic acid and Triton X-100 were all obtained from Sigma Chemical (St. Louis, MO).

Anti-nitrotyrosine antibodies used in immunofluorescence studies were kindly provided by Dr. H. Ischiropoulos, University of Pennsylvania. Fluorescein isothiocyanate-conjugate anti-rabbit IgG was obtained from Jackson Immunoresearch Laboratories Inc. (West Grove, PA). Donkey serum was from Sigma Chemical (St. Louis, MO).
2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Epithelial Cell Isolation and Culture

DLEC were harvested from late gestation fetal rats and grown in primary culture according to methods previously described (185). In brief, lungs were excised from 20 or 21 day GA (term = 22 d) Wistar rat fetuses and minced into 1-mm$^3$ pieces. The lung fragments were incubated at $37^\circ C$ with 0.125% trypsin and 0.002% DNase and dissociated cells were then passed through a Nitex 100 mesh filter (B. and S. H. Thompson, Scarborough, Ontario). The cells were then incubated with 0.1% collagenase and purified using differential adhesion techniques. These cells, of which >99% are epithelial as assessed by cytokeratin immunohistochemistry, are referred to as DLEC as they consist of mature and precursor ATII epithelial cells or distal airway cells (203). Cultured in this manner, they transport Na$^+$ via amiloride-sensitive and insensitive mechanisms (185) but have no detectable Cl$^-$ currents (268). Amiloride-sensitive NSC channels (193), as well as amiloride-sensitive Na$^+$ selective channels, have been demonstrated on the apical membranes of DLEC (255).

The harvested epithelial cells were immediately seeded onto 100 mm or 150 mm diameter tissue culture dishes (Falcon, Mississauga, Ontario), or tissue culture treated Snapwell® porous polycarbonate filters (Costar, Cambridge Mass, total surface area 1.13 cm$^2$). Seeding density was 3 x $10^5$ cells/cm$^2$ for tissue culture dishes, and 1 x $10^6$ cells/cm$^2$ for Snapwell® filters. All cells were grown in MEM with 10% FBS and
penicillin/streptomycin at 37°C in a humidified 95% air / 5% CO₂ environment.

Nonadherent epithelial cells were removed 24 hours after seeding. Epithelial monolayers used in Ussing chamber experiments were studied after 3 or 4 days in culture, while those used for total RNA extraction were grown to confluence for a minimum of 2 days prior to study. Cells used in immunofluorescence studies were seeded (3 x 10⁵ cells/cm²) onto 6 well plates (Costar) and studied 3-4 days later.

2.2.1.2 Alveolar Macrophage Isolation and Culture

Adult female Wistar rats (190-210 g) were anesthetized with halothane:nitrous oxide and then exsanguinated. The heart and lungs were extracted en bloc and the trachea cannulated (14 g catheter, Becton Dickinson Vascular Access, Sandy, UT). Ex vivo BAL was performed with five 10 ml aliquots of PBS with 1 mM ethylenediaminetetraacetate (EDTA). Fluid was recovered by gentle suction, and lavaged cells were pelleted by centrifugation (200 x g for 10 min), pooled, and resuspended in MEM containing 10% FBS and penicillin/streptomycin. Cell viability, as determined by trypan blue exclusion, was consistently >95%, and >92% of cells were AM, as demonstrated with Wright's staining. Total cell numbers were counted using a hemocytometer (Improved Neubauer, American Optical Corp., Buffalo, NY.) AM were then immediately cultured, either in the presence of DLEC (see below), or alone, for the purposes of supernatant acquisition. Those cultured alone were either plated on 100mm diameter tissue culture dishes (1x10⁷ cells/dish) in 10 ml of MEM with 10% FBS
and penicillin/streptomycin, or in 6 well plates (Costar, 2.25x10^5 cells/well) in 2.25 ml of the same medium, and exposed to 10µg/ml LPS for 18-24 hours. Cell-free supernatants, after centrifugation at 8000 g for 30 seconds, were then added to DLEC monolayers previously cultured in 100 mm tissue culture dishes or porous filters (see below).

All animals received humane care in compliance with the “Principals of Laboratory Animal Care” formulated by the National Society for Medical Research, the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Science and published by the National Institute of Health (NIH Publication No. 86-23), and the “Guide to the Care and Use of Experimental Animals” formulated by the Canadian Council of Animal Care. All protocols were approved by the Animal Care Committee of the Toronto Hospital (Toronto General Division) and The Hospital for Sick Children Animal Research Ethics Committee.

2.2.2 Northern Analysis

DLEC total RNA was extracted using the method of Chomczynski and Sacchi (45). After spectrophotometric quantitation, 15 µg of total RNA underwent electrophoresis in a 1% agarose gel containing formaldehyde, blot-transferred to a Hybond-N* nylon membrane (Amersham, Buckinghamshire, UK), baked at 80°C for 10 minutes and then UV crosslinked. Membranes were then pre-hybridized and hybridized
with solutions containing 50% deionized formamide, 5x Denhardt's solution, 1% SDS, 5x SSC and 0.2 mg/ml herring sperm DNA. Hybridization was performed overnight with 32P random-labeled (Multiprime DNA kit, Amersham) cDNA probes for one or more of α, β, and γ rENaC (35,37). Blots were then washed using a final solution consisting of 0.1x SSC and 0.1% SDS at 50°C for 30 minutes. Transcripts were visualized using standard autoradiography and/or phosphor-imaging. After stripping the blots in a solution containing 1 mM TRIS HCl, 1 mM EDTA, and 20 % SDS, RNA integrity and comparable RNA loading between lanes was assessed by hybridization of membranes with a cDNA probe for 18S RNA (American Type Culture Collection, Rockville, Maryland). Final wash utilized 0.1x SSC and 0.1% SDS at 65°C for 15 minutes.

2.2.3 Bioelectric Properties of the DLEC Monolayer

DLEC monolayers cultured on Snapwell® filters were studied by placing them in Ussing chambers (MRA, Clearwater, FL) that contained warmed HBSS and 22.4 mM sodium bicarbonate (NaHCO3) which was circulated using an air lift of 95% air/5% CO2 gas mixture. Measurements of the monolayer's bioelectric properties were made with KCl agar-calomel half-cells and silver-silver chloride electrode-saline agar bridges that were connected to a high impedance millivoltmeter that could function as a voltage-current clamp with automatic fluid resistance compensation (VCC 600 Physiologic Instruments, San Diego, CA). The transepithelial PD was recorded continuously under
open circuit conditions using a linear chart recorder (Linear Recorder 585, Baxter, Toronto, Canada). Every 10 seconds a 0.5 second duration 1 μA pulse of current was delivered across the monolayer so that the measured change in PD enabled calculation of $R$ using Ohm's law. After stabilization of PD, both at baseline and after the addition of various ion transport inhibitors (see below), transepithelial PD was temporarily clamped to 0 mV so that $I_{ac}$ could be recorded. At the end of each experiment, ethylenediaminetetraacetate (EDTA) was added to both surfaces of the monolayer and previous measurements corrected for the observed residual PD.

2.2.4 Nitrite Analysis

NO decomposes rapidly in the presence of $O_2$ and $H_2O$ to yield nitrite and nitrate as stable end products. Nitrite levels thus reflect NO production from all sources. The nitrite content of cell-free supernatants from appropriate experiments was measured at the end of the coculture period by reacting samples (100 μl) with the Griess reagent (100 μl) for 10 min at 37°C according to established methods (87). Absorbance at 540 nm was then measured and nitrite concentrations were calculated from a linear standard curve generated between 0 and 128 mM sodium nitrite.

2.2.5 Detection of Nitrotyrosine Residues

Cells were cultured on glass cover slips in 6-well tissue culture dishes. Cover slips were then washed three times with ice cold PBS and fixed in 100% methanol (-20°C, 10 min). Cover slips were blocked in 5% donkey serum-PBS for 2 h at room temperature, then incubated in primary antibodies overnight at 4°C (titre of 1:100 diluted in 1% BSA-PBS). To demonstrate the specificity of immunofluorescence, antibodies were co-incubated with the
antigenic fusion protein (1 mg fusion protein/mg of antibody) in a total volume of 100 μl of antibody buffer (1% BSA-PBS) overnight at 4°C on a rotating shaker. Coverslips were then washed three times in ice cold PBS, and secondary antibodies were applied (6 μg/ml fluorescein labeled anti-rabbit IgG) for 2 h at room temperature. Cells were washed as before and mounted for confocal microscopic visualization using Slow Fade (Molecular Probes Inc., Eugene, OR).

2.2.6 DLEC Cytotoxicity

Confluent epithelial monolayers in primary culture, as described above, were preincubated with 0.2 mCi/ml [14C]adenine for 2 hours. DLEC cytotoxicity resulting from specific interventions (see below) was assessed by measuring [14C]adenine levels in cell-free supernatants. At the end of each experiment, epithelial cell monolayers were lysed with Triton X-100 (0.1%) and collected. The percentage of [14C]adenine release during culture was calculated after determination of total radioactivity both in the supernatant and the cell lysate by liquid scintillation counting (1219 Rack Beta Liquid Scintillation Counter, LKB Wallac, Sweden).
2.3 Experimental Protocols

2.3.1 Aim 1: Effect of products of LPS-stimulated AM on DLEC rENaC mRNA expression.

To determine if soluble factor(s) released from endotoxin-stimulated AM could modulate DLEC α-, β-, and γ-rENaC mRNA levels, DLEC monolayers cultured for a minimum of 2 days in 100 mm tissue culture dishes were exposed to either AM alone (1 x 10^6 cells/ml media), LPS (10 μg/ml) alone, or AM and LPS together. Control groups consisted of DLEC exposed to culture medium alone. At various time points, DLEC monolayers were washed vigorously with cold, Ca^{2+}-Mg^{2+}-free HBSS, RNA was extracted, and northern blot analysis performed.

Similar experiments were performed involving the physically separated coculture of DLEC and AM. A 60 mm diameter tissue culture dish was secured with silicone in the center of a larger, 150 mm diameter tissue culture dish (figure 7). DLEC were seeded and grown to confluence over at least a 2 day period in the outer well. AM (1.0 x 10^7) were then added to the central well and allowed to adhere for 30 minutes at 37°C. Additional culture medium was then added so that common medium bathed both the inner and outer wells. LPS (10 μg/ml) was added to the system and gentle agitation of these dishes allowed circulation of medium without the displacement of adherent AM into the outer well. Control groups consisted of DLEC alone. At various time points, DLEC total RNA was extracted and northern blot analysis performed.
Studies performed previously in our laboratory have demonstrated no evidence of DLEC cytotoxicity resulting from 16 hours of direct coculture with LPS-stimulated AM. In order to ensure that such exposure had not in fact initiated a process that would ultimately lead to cell death, similar studies were performed in the physically separated coculture system at later time points, after removal of LPS-stimulated AM-conditioned medium. Confluent epithelial monolayers grown in the outer well of tissue culture dishes, as described above, were exposed to soluble factors released from LPS-stimulated AM grown in the inner well (1 x 10^7 AM). Coculture in common bathing medium was continued for 16 hours. Cell-free supernatants were collected and sufficient media added to bathe only the epithelial cells in the outer well, thereby effectively ending coculture. Culture of DLEC alone was continued for a further 24
hours. Cell-free supernatants were again collected and [14C]adenine release at both
time points was then determined as an index of DLEC injury.

2.3.2 Aim 2: Modulation of altered DLEC rENaC mRNA expression.

Since our experiments demonstrated a decrease in α-, β-, and γ-rENaC mRNA
expression as a result of exposure to a soluble factor(s) released from LPS-stimulated
AM, experiments were undertaken to investigate how this reduction in steady state
mRNA levels could be prevented. To determine if the inhibition of NO production could
modulate this effect, experiments were undertaken involving direct coculture of DLEC
and LPS-stimulated AM, with and without the addition of the competitive NOS inhibitor,
L-NMMA (0.1 mM). Coculture was continued for 8 hours, after which DLEC total RNA
was extracted and northern analysis performed. NMMA was added at the beginning of
coculture and again at 4 hours. In subsequent experiments, total RNA from DLEC
cultured for 8 hours in the presence of SNAP, a non-enzymatic NO donor, was similarly
examined. SNAP (0.1 mM) was also added at 4 hour intervals, for a total of 2 doses. In
order to determine if these agents, SNAP and NMMA, had been effective in generating
and inhibiting the synthesis of NO, respectively, nitrite analysis was performed on cell-
free supernatants from these experiments.
2.3.3 Aim 3: Role of peroxynitrite in the LPS-stimulated AM-induced impairment of DLEC Na\(^+\) transport.

Given that peroxynitrite is a metabolic product of NO that exhibits strong reactivity with protein thiols, experiments were carried out to detect the possible presence and action of this ROS in the experimental model. In these studies, DLEC were cultured on cover slips in direct contact with AM for 16 hours, with or without LPS (10 \(\mu\)g/ml). Confocal immunofluorescence studies were then performed with the cocultured cells (DLEC and AM) using anti-nitrotyrosine antibodies.

2.3.4 Aim 4: Effect of NAC in modulating the LPS-stimulated AM-induced impairment of DLEC Na\(^+\) transport.

In order to determine if NAC could modulate the change in DLEC amiloride-sensitive Na\(^+\) transport induced by soluble AM products after LPS-stimulation (48), experiments were conducted using NAC. Initial studies were performed to confirm the previously observed effect of exposure to LPS-stimulated AM supernatant on DLEC bioelectric properties (48). Monolayers, in culture for 3-4 days on porous filters, were exposed to either medium alone or the cell-free supernatant of LPS (10 \(\mu\)g/ml)-stimulated AM (2.25 \(\times\) 10\(^5\) cells/well), cultured for 18-24 hours in 6-well plates, as described above. At various time points, filters were mounted in Ussing chambers. After determining baseline bioelectric properties, the Na\(^+\) transport blocker amiloride (0.1 mM apically), the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransport inhibitor bumetanide (0.1mM basally) and flufenamic acid (0.45 mM apically), which inhibits both the NSC and Ca\(^{2+}\)-activated
Cl- channels, were added sequentially to the monolayer. This enabled the calculation of amiloride-sensitive and insensitive current, as a percentage of total current, in all experimental groups.

Subsequent studies were carried out with the addition of NAC to this supernatant coculture system. DLEC monolayers and cell-free LPS-stimulated AM supernatant were prepared as described above. Study groups included control monolayers exposed to medium alone, and DLEC exposed on their apical surfaces to the supernatant of LPS-stimulated AM in the presence and absence of NAC (20 mM). In the latter groups, NAC was added to the apical side of the monolayers. At various time points following the first dose of NAC, the apical bathing media from all study filters was removed and replaced with either an equal volume of fresh media or LPS-stimulated AM supernatant. In NAC-treated groups, a second dose of NAC was added. At given time points, monolayers were mounted in Ussing chambers and studied as described above.

To determine if the observed effect of NAC might be related to an increase in intracellular thiol levels, particularly those of glutathione, experiments were performed using dithiothreitol (DTT) instead of NAC. DTT is also a thiol antioxidant which enters cells less readily than NAC and, in this experimental system, acts predominantly extracellularly (see discussion). Only one dose of DTT (100 μM) was added at the initiation of supernatant coculture, and monolayers were examined in the Ussing chamber at 8 and 16 hours.
Statistical analysis Results are presented as mean ± 1 standard deviation (SD) of n experiments unless otherwise indicated. The statistical significance of differences between the means of multiple groups or the means of an individual group at multiple time points was determined by one-way ANOVA followed by Newman-Keuls multiple intergroup comparisons. Student's unpaired two-tailed t test was used to assess significance between two groups. Probability (p) <0.05 was considered statistically significant.
3.1 Aim 1: Effect of products of LPS-stimulated AM on DLEC rENaC mRNA expression.

DLEC exposed to control medium only, as well as AM or LPS alone, expressed significant levels of α-, β-, and γ-rENaC, as illustrated in figure 8. rENaC expression was significantly decreased, however, in DLEC exposed to LPS-stimulated AM for 16 hours in the direct coculture system. Similar results were obtained after 16 hours of physically separated coculture (data not shown), implicating a role for a soluble factor(s) released from AM exposed to LPS. DLEC exposed to LPS alone for 16 hours showed persistent strong rENaC mRNA expression, although this was decreased slightly in comparison to control cells (71% of control values).

Studies were undertaken to examine changes in DLEC rENaC mRNA expression over time following both direct and physically separated coculture with LPS-stimulated AM. A representative example of the time course of this effect on α-rENaC mRNA expression, in physically separated coculture, is shown in figure 9. A clear effect was also consistently demonstrated after 8 hours of direct coculture (data not shown).
Figure 8. Effect of direct LPS-stimulated AM coculture on DLEC rENaC mRNA expression. The level of α-, β-, and γ-rENaC mRNA expression in DLEC cultured for 16 hours in the presence or absence of AM with or without LPS (direct coculture). Control monolayers were exposed to media only. Expression of 18S RNA was used to assess RNA integrity and loading. rENaC mRNA expression was significantly decreased in DLEC exposed to LPS-stimulated AM. These data are representative of 3 separate studies.
Figure 9. Time course of changes in DLEC α-renac mRNA expression resulting from physically separated coculture with LPS-stimulated AM. The level of α-renac mRNA expression over time in DLEC physically separated from LPS-stimulated AM, as described above. α-renac mRNA expression was consistently decreased after 8 hours of coculture in the presence of LPS. These data are representative of 4 separate time course studies.
DLEC cytotoxicity DLEC exposed to LPS-stimulated AM in physically separated coculture for 16 hours showed no significant difference in [14C]adenine release as compared to cells exposed to medium alone (figure 10). Similarly, no evidence of DLEC cytotoxicity was seen 24 hours after the removal of the medium conditioned by the activated AM (figure 10).

Figure 10. Effect of LPS-stimulated AM coculture on DLEC [14C]adenine release. [14C]adenine release from DLEC after 16 hours of physically separated coculture with AM in the presence of LPS (open bars) and 24 hours after termination of coculture (black bars), shown as a percentage of total release resulting from Triton-X treatment. Control DLEC were exposed to medium alone. No increase in [14C]adenine release was seen from cocultured DLEC. n = 3 samples per group. p > 0.05 for all groups by ANOVA.
3.2 Aim 2: Modulation of altered DLEC rENaC mRNA expression.

*Role of NO in changes in rENaC mRNA expression*  DLEC were cocultured with LPS-stimulated and non-stimulated AM. Again, LPS-stimulated AM caused a significant decrease in rENaC mRNA expression after 8 hours of direct coculture. As shown in figure 11a, the addition of NMMA (0.1 mM) at the start of coculture, and again at 4 hours, did not modulate this effect, despite a significant decrease in NO production. Nitrite levels in cell free supernatants from DLEC cocultured with AM alone were not significantly different than those from DLEC exposed to medium alone (Figure 11b). There was a significant increase in NO production, as reflected by increased nitrite levels, arising from DLEC coculture with AM and LPS, which was prevented by the addition of NMMA (Figure 11b). This indicated that the blockade of NO generation did not prevent the decrease in rENaC mRNA expression during direct coculture of DLEC with LPS-stimulated AM. Consistent with this, Figure 12a shows a representative northern blot from experiments using the non-enzymatic NO donor, SNAP. Despite a significant increase in NO production (Figure 12b), SNAP (0.1 mM) treatment at 4 hour intervals, for a total of 8 hours, did not result in a decrease in rENaC mRNA levels.
Figure 11. Effect of NMMA on coculture-induced decrease in DLEC rENaC mRNA expression.

A. DLEC were cultured for 8 hours in the presence of LPS-stimulated AM with or without NMMA (0.1 mM). Control monolayers were exposed to AM in the absence of LPS. NOS inhibition failed to prevent the decrease in rENaC mRNA expression. These data are representative of 3 separate studies.

B. Nitrite levels in the cell free supernatants of DLEC exposed to media alone, DLEC in the presence of AM with and without LPS, and DLEC exposed to LPS-stimulated AM with NMMA. NMMA significantly reduced supernatant nitrite levels. n = 6 samples per group, *p < 0.001 vs. all other groups by ANOVA.
Figure 12. Effect of SNAP on DLEC rENaC mRNA expression.
A. DLEC were exposed to SNAP (0.1 mM) for 8 hours. SNAP treatment did not affect rENaC mRNA expression. These data are representative of 3 separate studies.
B. Nitrite levels in the cell-free supernatants of DLEC exposed to SNAP. SNAP treatment significantly increased supernatant nitrite levels. n = 6 samples per group. *p < 0.001 vs. DLEC alone by 2-sided Student t test.
3.3 Aim 3: Role of peroxynitrite in the LPS-stimulated AM-induced impairment of DLEC Na+ transport.

Immunofluorescence studies using an anti-nitrotyrosine antibody were performed to detect the action of peroxynitrite in our system. In order to define the subcellular distribution of nitrotyrosine in cocultured DLEC and AM, confocal immunofluorescence was performed (figure 13a). When scanned in the xy-plane, there was no nitrotyrosine staining in isolated DLEC (figure 13a, left panel). By contrast, when DLEC were cocultured with AM, there was marked AM staining (figure 13a, middle panel). The distribution of this staining was intracellular, with a discrete punctate appearance suggestive of a vesicular or granular compartment. In the presence of endotoxin, the degree of AM staining was increased, although the subcellular distribution did not change (figure 13a, right panel). Importantly, the conditions of coculture with endotoxin-stimulated AM did not alter the presence of nitrotyrosine in the DLEC (figure 13a, right panel). The specificity of the immunodetected nitrotyrosine was confirmed by the absence of signal in cocultured AM incubated with either the secondary antibody alone, or when the primary antibody was pre-incubated with the cognate fusion protein (Figure 13b).
Figure 13. Detection of nitrotyrosine residues by immunofluorescence.
A. No significant nitrotyrosine staining is detected in DLEC exposed to medium alone, nor in those exposed to alveolar Mφ with or without LPS, for 16 hours. Marked staining is seen, however, in alveolar Mφ cocultured with DLEC (middle panel), which is increased in the presence of LPS (right panel).
B. Specificity of immunodetected nitrotyrosine is confirmed by the lack of staining in the presence of secondary antibody alone (middle panel) or with preincubation of primary antibody with the cognate fusion protein (right panel).
Phase

DLEC

DLEC + MΩ

DLEC + MΩ + LPS

Confocal α-nitrotyrosine
3.4 Aim 4: Role of NAC in modulating LPS-stimulated AM induced impairment of DLEC Na⁺ transport.

When DLEC were exposed to the supernatant of LPS-stimulated AM, there was a significant decrease in DLEC amiloride-sensitive $I_{sc}$, as compared to DLEC exposed to medium alone (figure 14). NAC (20 mM), given 1 hour prior to the beginning of coculture, and again when supernatants were added to DLEC monolayers, partially reversed this impairment during 16 hours of exposure (figure 14).

![Figure 14. Effect of N-acetylcysteine on AM supernatant (SUP)-induced reduction of amiloride-sensitive $I_{sc}$. NAC (20 mM) partially prevented the reduction of DLEC amiloride-sensitive $I_{sc}$ resulting from exposure to the supernatant of LPS-stimulated AM. $n = 5-11$ samples per group. *p < 0.001 vs. DLEC alone by ANOVA.](image-url)
Baseline bioelectric properties, as summarized in table 8, were not different between experimental groups.

**Table 8. DLEC Baseline Bioelectric Properties**

<table>
<thead>
<tr>
<th></th>
<th>DLEC (n = 5)</th>
<th>DLEC + Sup (n = 5)</th>
<th>DLEC + Sup + NAC (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Isc (µA/cm²)</strong></td>
<td>4.32 ± 0.26</td>
<td>4.66 ± 1.02</td>
<td>4.85 ± 0.71</td>
</tr>
<tr>
<td><strong>Resistance (Ω·cm²)</strong></td>
<td>1993 ± 391</td>
<td>2011 ± 805</td>
<td>2049 ± 495</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD
p > 0.05 for all groups by ANOVA

Addition of dithiothreitol (DTT, 100 µm) did not prevent the impairment of DLEC amiloride-sensitive I_{sc} resulting from 16 hours of exposure to LPS-stimulated AM supernatant (Figure 15).
Figure 15. Effect of dithiothreitol on AM supernatant (SUP)-induced reduction of amiloride-sensitive $I_{sc}$. DTT (100 μM) failed to modulate the impairment of DLEC $I_{sc}$ resulting from exposure to the supernatant of LPS-stimulated AM. $n = 4-6$ samples per group. *$p < 0.001$ vs. DLEC alone by ANOVA.
DISCUSSION

The ability of the alveolar epithelium to maintain vectorial Na⁺ transport represents an important property of the alveolar-capillary barrier, which helps maintain a fluid-free alveolar space. Our laboratory has previously demonstrated that soluble product(s) from AM stimulated with bacterial endotoxin impair DLEC Na⁺ transport through an L-arginine dependent mechanism (48), an effect that is reproduced directly by NO (56). The present studies show that coculture with LPS-stimulated AM results in decreased DLEC gene expression for a specific epithelial Na⁺ channel, rENaC. This represents a distinct process that does not depend on the generation of NO, nor did we find histologic evidence supporting an important role for peroxynitrite in the L-arginine dependent impairment of DLEC Na⁺ transport resulting from coculture. Furthermore, these studies confirm that the cell-free supernatant from LPS-stimulated AM also impairs DLEC Na⁺ transport, a process previously shown to be unaffected by treatment with NMMA (48). NAC modulates this effect, likely through a specific effect on intracellular levels of glutathione. These data, therefore, represent further evidence for the potential role of impaired DLEC Na⁺ transport in ALI, and suggest a possible strategy to modulate it, using NAC.

A profound decrease in mRNA levels for all three rENaC subunits was seen as a result of both direct and physically separated coculture of DLEC with LPS-stimulated AM. The latter observation showed that the process was mediated by soluble AM

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products and was not dependent on direct cell contact. This was consistent with previous studies in our laboratory showing that physically separated coculture resulted in a decrease in DLEC $I_{sc}$, as well as an increase in DLEC iNOS gene expression (56).

This effect on Na$^+$ channel gene expression is not related to cytotoxicity, resulting in a general depression of cell function. This is shown by previous $[^{14}\text{C}]$adenine release experiments (48), and confirmed in the present studies. Furthermore, it is demonstrated here that coculture does not initiate a process leading ultimately to cell death, since no increase in $[^{14}\text{C}]$adenine release is seen in DLEC 24 hours after the end of coculture. Since coculture resulted in an increase in DLEC iNOS mRNA expression, and stable levels of 18S RNA, the decrease in rENaC RNA levels cannot be explained by a general depression of DLEC mRNA synthesis resulting from coculture or by unequal loading or RNA degradation in northern blot analyses.

These changes in rENaC mRNA levels resulting from coculture represent a process with characteristics apparently distinct from the previously described alterations in DLEC bioelectric properties. Both the decrease in DLEC amiloride-sensitive $I_{sc}$ (48) and the modulation of rENaC mRNA levels resulting from coculture occurred between 4 and 8 hours after the initiation of coculture. The temporal relationship between alterations in mRNA expression and the reflection of these changes in functional channel activity would depend on several factors, including translational regulation and the extent of post-translational modification, as well as the existing intracellular pool of channel proteins and, particularly, the half-life of rENaC in epithelial membranes. Patch clamp studies done previously in our laboratory revealed a selective decrease in the
membrane density of the amiloride sensitive 25 pS NSC channel resulting from 16 hours of direct coculture with LPS-stimulated AM (48), an effect reproduced by SNAP (56). Although the true functional characteristics of ENaC in cell membranes is unknown (see below), it is widely held that rENaC represents a highly Na⁺ selective channel (78), given the highly selective nature and low conductance characteristics of Na⁺ transport in oocytes expressing the rENaC subunits (37). The number of 12 pS Na⁺ selective channels in our DLEC were unaffected by this coculture protocol, despite the significant decrease in amiloride-sensitive \( I_{sc} \) (48). Further evidence suggesting that the change in rENaC mRNA expression is not part of the same process affecting DLEC bioelectric properties is seen when the results of the experiments using NMMA and SNAP are considered. Given the importance of NO in the alterations in bioelectric properties, its role in the modulation of rENaC gene expression was investigated. The failure of NMMA to prevent the decrease in \( \alpha \), \( \beta \), and \( \gamma \)-rENaC mRNA levels resulting from coculture with LPS-stimulated AM, as well as the inability of SNAP to recapitulate this effect, supported the assertion that the effects on bioelectric properties and on ENaC gene expression were distinct processes.

The functional significance of alterations in DLEC rENaC mRNA expression in this context is unknown. The contribution of ENaC to \( \textit{in vitro} \) bioelectric properties of DLEC, specifically \( I_{sc} \), has not been clarified. As indicated above, the behaviour of ENaC has been largely inferred from studies involving cellular expression systems, and the structure and characteristics of ENaC in native lung epithelia is largely unknown. Work by Ismailov and colleagues have indicated that \( \alpha\text{-}\beta\text{-ENaC} \) (bovine) may well
represent an important core component of the amiloride-sensitive Na⁺ channel immunopurified from bovine renal papillae (117). In their study, polyclonal antibodies raised against an α-γENaC fusion protein recognized a 70 kDa polypeptide component of the previously characterized renal Na⁺ channel. This protein complex, as described above, consists of at least 6 different subunits (5,18). The immunopurified channel, when reconstituted in planar lipid bilayers, displays functional characteristics which are significantly different from those seen in oocytes expressing the rENaC subunits. This includes the presence of different, discrete conductance states, including a 40 pS main state (116). These conductance properties could be modulated by disulfide bond reduction with DTT, and consequent dissociation of protein complexes, resulting in biophysical characteristics more comparable to those seen when ENaC alone was reconstituted in lipid bilayers (115). Notably, the cation selectivity of the DTT-treated immunopurified Na⁺ channels is lost in response to stretch (117). These data suggest that associated channel proteins, as well as dynamic cellular characteristics, mimicked by stretch, might significantly modulate the characteristics of the channel complex of which ENaC is a component, making the actual in vivo behaviour of ENaC difficult to predict. It must be noted, however, that the data from Ismailov and colleagues (116,117) and other workers must be interpreted with caution as the effects of protein purification and the presence of artificial rather than native membranes in the bilayer may have influenced the biophysical properties of the reconstituted channels.
In keeping with this, the consequences of changes in expression of ENaC to in vivo alveolar fluid clearance in the setting of pathological pulmonary edema is not completely understood. Compelling evidence does exist, however, regarding the importance of ENaC in the clearance of fetal lung fluid at the time of birth. The expression of ENaC subunits is developmentally regulated and correlates with the appearance of the capacity for Na⁺ absorption, as opposed to Cl⁻ secretion, in fetal whole lung and its cultured epithelial cells (184,252). A recent study described the perinatal course of mice in which the α-ENaC (mouse) gene had been inactivated by gene targeting (109). All affected neonates developed respiratory distress and died shortly after birth, despite otherwise normal morphologic lung development. Whole lungs from these mice had significantly higher wet-to-dry weight ratios as compared to wild-type littermates and showed a pattern similar to that seen with previous pharmacologic blockade of Na⁺ channels in newborn guinea pigs (185,186). Amiloride-sensitive electrogenic Na⁺ transport was abolished in the epithelium of excised tracheal cysts from affected mice. Neonates retaining one normal α-ENaC gene copy were phenotypically and electrophysiologically normal. These results show that α-ENaC gene expression in the mouse is vital for normal perinatal lung liquid clearance and the adaptation to ex utero life.

Specific mutations of human ENaC (hENaC) subunit genes also produce well defined phenotypic effects. Pseudohypoaldosteronism type I (PHA I) is a rare disease manifesting in the neonatal period and characterized by salt wasting with dehydration, hyponatremia, hyperkalemia, metabolic acidosis, failure to thrive and weight loss.
despite normal adrenal function. Both autosomal dominant and recessive inheritance patterns have been described. In 5 of 7 kindreds analyzed, mutations resulting in a disruption of protein structure prior to the second putative transmembrane segment were found in genes coding for α- and β-subunits of ENaC (42). Surprisingly, given the results from the α-mENaC knockout mice experiments, these individuals do not suffer from RDS at birth. This may indicate the existence of alternative or additional Na⁺ conductive channels in the human distal lung epithelium which could compensate for the absent ENaC. This is perhaps comparable to the situation encountered in experimental models of cystic fibrosis. Cystic fibrosis transmembrane conductance regulator (CFTR) knock-out mice of mixed genetic background demonstrate defective nasal epithelium but do not develop lung disease, despite the genetic defect responsible for clinical lung disease in humans. This is likely the result of the compensatory action of a Ca²⁺-activated Cl⁻ channel in the lower airways of mice.

Mutations of the hENaC gene can also lead to increased channel function, resulting in well-defined clinical manifestations. Liddle’s syndrome is an inherited, autosomal dominant form of hypertension characterized by abnormal activation of the amiloride-sensitive Na⁺ channel in the epithelium of the distal nephron of the kidney. This results in excessive Na⁺ absorption despite low aldosterone levels and suppressed plasma renin activity. Linkage of the β-hENaC gene with the disease in the original kindred was demonstrated, and further study revealed a premature stop codon that truncates the cytosolic carboxy-terminus of the β-subunit in affected individuals (227). Similar mutations were also documented in the γ-subunit in some affected people (97).
Expression of the cRNA of such abnormal subunits in Xenopus oocytes resulted in increased Na\(^+\) current with no change in channel conductance or P\(_o\), suggesting an increased channel number in the membrane, possibly resulting from defective channel internalization.

The AM product(s) involved in the changes in rENaC mRNA expression were not identified in these studies. As indicated above, activated M\(_{\phi}\) release a wide range of inflammatory mediators which may play a role in these effects. Prominent among these are the cytokines, which are potent, bioactive molecules and important mediators of the host response to inflammatory stimuli, particularly sepsis (225). The best studied, and perhaps most important of these, are TNF-\(\alpha\) and IL-1, both of which are released from M\(_{\phi}\) in response to LPS exposure (275). These and other cytokines are able to exert a myriad of biological effects, including influencing the temperature set point, vascular resistance and permeability, and cardiac and bone marrow function (225). Studies examining patients with ARDS have shown significantly higher BAL fluid cytokine levels as compared to normal controls and to those at risk for the development of ARDS (111,249). Persistent elevation of BAL cytokine levels may be associated with a poorer outcome (162). Much evidence exists for the ability of such cytokines to influence gene expression. A classic example of this is the acute-phase response, which is characterized by changes in concentrations of a wide variety of plasma proteins in response to inflammation or tissue injury (131). In vitro studies have indicated that this largely involves an increase in hepatic synthesis of a number of proteins, including C-reactive protein, complement proteins, proteinase inhibitors, components of the
coagulation cascade and metal binding proteins (196). Of note, synthesis of several
proteins decreases during the acute phase response, including albumin, the iron
binding protein transferrin, and apolipoprotein A-1 (196). It is recognized that these
processes are mediated in large part by a variety of cytokines, including IL-1 and TNF
(130). Such regulation appears to occur primarily on a transcriptional level in
hepatocytes, involving cytokine-induced nuclear transcription factors (196). These
proteins, including activator protein (AP)-1 and nuclear factor (NF) \( \kappa \)B, bind to promoter
regions of specific genes and act to augment, or in the case of other factors, to
suppress, transcription (99). In addition to transcriptional regulation, studies have
shown that modulation can also occur at a post-transcriptional level, involving altered
mRNA processing, changes in translational efficiency, and effects on protein
modification and export (196).

ROS are also recognized to modulate gene expression. Oxidant stress
increases expression of a variety of antioxidant enzymes and scavenging proteins in
the lung and in lung epithelial cells, including MnSOD and glutathione peroxidase (34),
a response clearly designed to restore cellular homeostasis. This also involves
transcriptional regulators, particularly AP-1 and NF-\( \kappa \)B (34).

In addition to identifying and describing the effect of coculture with LPS-
stimulated AM on DLEC \( \alpha \)EnaC expression, experiments were undertaken to clarify
mechanisms responsible for the apparently distinct effect of this model on DLEC
bioelectric properties. As indicated, direct or physically separated coculture of DLEC
with endotoxin-stimulated rat AM results in a decrease in epithelial Na\(^+\) transport, a
process dependent, in part, on the generation of NO. As outlined above, NO is recognized to act through a number of mechanisms, including the activation of guanylate cyclase, the formation of peroxynitrite, and through interaction with protein thiols.

Although cGMP-mediated events have been shown to influence inner medullary collecting duct (IMCD) cell NSC channel function (139-141), previous studies in our laboratory failed to show any effect of either a membrane permeant cGMP analogue or ANP, which increases intracellular cGMP levels, on fetal DLEC bioelectric properties (187). DLEC were, however, exposed to these agents for a maximum of only 30 minutes, considerably shorter than the 6-8 hours necessary to see any effect of coculture on fetal DLEC bioelectric properties. The role of prolonged stimulation of the cGMP pathway in this process, therefore, remains unresolved.

Several lines of evidence suggest a role for peroxynitrite in ALI. Human lung surfactant is damaged by peroxynitrite (91), a characteristic of ARDS. The presence of nitrotyrosine residues, the product of the nitrosylation of the hydroxy group of this amino acid by peroxynitrite, has been demonstrated in lung sections from rats exposed to hyperoxia and following intravenous administration of endotoxin (92,277). Similar findings were demonstrated in lung sections from patients with ARDS (92). Of note, peroxynitrite also resulted in an impairment of Na\(^+\) transport in alveolar epithelial cells, as reflected by a decrease in \(^{22}\text{Na}^+\) uptake across adult ATII cell monolayers cultured from rabbits (107). Given these data, it was possible that peroxynitrite represented an important mediator of the coculture-induced impairment of DLEC Na\(^+\) transport.
Consideration of our previous results, showing that SNAP alone could reproduce this effect (56), argues against this. SNAP is considered to release NO alone, in contrast to 3-morpholinosydnonimine (SIN-1), which simultaneously generates NO and \( \text{O}_2^- \) (112). While the *in vitro* exposure of the surfactant protein SP-A to SIN-1 resulted in an impairment of this protein's ability to enhance lipid aggregation, likely through generation of peroxynitrite, SNAP had no such effect (91). This, of course, did not preclude the possibility that SNAP was reacting with \( \text{O}_2^- \) produced by the DLEC themselves. The studies described here, however, showing the failure of 16 hours of direct coculture to cause any significant detectable increase in the number of nitrotyrosine residues on the membranes of cocultured DLEC, as compared to DLEC exposed to medium alone, is more definitive evidence arguing against an important role for peroxynitrite in this model. The presence of nitrotyrosine residues on the membranes of LPS-stimulated AM does reflect production of NO and \( \text{O}_2^- \) by these cells.

The potential role of RS-NO formation in mediating the effects of coculture on DLEC \( \text{Na}^+ \) transport were not specifically investigated in these studies. As noted above, the generation of these compounds is recognized to underly a number of important cellular processes (239). In the airways, albumin and glutathione represent the predominant thiols, and, therefore, substrates for RS-NO formation (79). S-nitrosoglutathione (GS-NO) is a stable adduct of NO and has been shown to be present in the airways of normal individuals (80). A role for RS-NO formation in the impairment of membrane ion fluxes has been suggested by studies examining the neurotoxic
effects of NO (144). Lipton and colleagues examined the effects of NO generation under different redox conditions on the function of the N-methyl-D-aspartate (NMDA) receptor, the activation of which leads to Ca\(^{2+}\) influx into neurons, with resultant cell death. It was found that when conditions favoured the generation of peroxynitrite, neurotoxicity was seen. NO-mediated neurotoxicity was reversed, however, in redox conditions favouring the formation of RS-NO compounds. This was thought to be related to S-nitrosylation, and consequent down-regulation, of the NMDA receptor, resulting in attenuation of intracellular Ca\(^{2+}\) influx.

Finally, these studies demonstrate that NAC can partially reverse the impairment of DLEC Na\(^{+}\) transport resulting from exposure to the supernatant of LPS-stimulated rat AM. The supernatant coculture model was chosen in order to specifically examine the effect of NAC treatment on the DLEC, as opposed to the AM. The results of the present studies confirm our previous observation (48) that the supernatant of endotoxin-treated rat AM can reproduce the impairment of DLEC amiloride-sensitive Na\(^{+}\) transport caused by coculture with the activated AM themselves. The amiloride-sensitive \(I_{sc}\) likely reflects blockade of Na\(^{+}\) channels, as our laboratory has previously shown that amiloride blocks 12 pS Na\(^{+}\) selective and 25 pS NSC channels (149, 193) and whole cell Na\(^{+}\) currents (268) in the apical membrane of these epithelial cells. Further, dimethylamiloride, an amiloride analogue with high potency for the Na\(^{+}/H^{+}\) antiport, does not affect \(I_{sc}\) in vivo lung water clearance (185). DLEC \(I_{sc}\) (188) or whole cell Na\(^{+}\) currents (268). Treatment of DLEC with NAC during supernatant exposure resulted in a consistent, significant modulation of this effect by 16 hours. The fact that this modulatory action, although
evident, was less pronounced at 8 hours, raises the possibility that NAC may accelerate recovery of functional Na\(^+\) transport, as opposed to preventing the impairment from developing.

The specific mechanisms by which conditioned supernatant influences DLEC Na\(^+\) transport remain to be clarified. This obviously involves a soluble, AM-derived mediator with a half-life sufficiently long to result in effects after supernatant transfer. Given the extremely short half-life of NO in aerobic culture conditions (79), AM-derived NO cannot directly mediate the changes in DLEC Na\(^+\) transport. It has been proposed, however, that the formation of RS-NO compounds might represent a mechanism by which the bioactivity of NO could be preserved and its potential oxygen-dependent toxicity limited. In plasma, free NO exists only at nanomolar concentrations, whereas RS-NO compounds are detected at micromolar levels, predominantly as an adduct with serum albumin, the most abundant plasma protein thiol (240). The RS-NO compound formed by the reaction of NO with bovine serum albumin exerted much longer lived, although less potent, vascular effects as compared to compounds directly releasing NO (127). These data all reflect the significantly longer half-life of RS-NO compounds, as compared to free NO (241). Similar evidence exists supporting a biologically important role for RS-NO formation in human airways (80). Gaston and colleagues showed that RS-NO compounds, predominantly the adduct with glutathione, exist in the BAL fluid from normal human lungs, and that their levels increase in certain pathophysiologic states, including pneumonia and the post-transplant lung. This likely reflects increased NO production, supported by the profound increase in BAL fluid RS-NO levels resulting
from inhalation of NO gas. Notably, endogenous RS-NO compounds are considerably more stable than free NO in the airways, with a half-life in 21% O₂ of approximately 3 hours. Exogenous S-nitrosoglutathione (GS-NO) behaved similarly. This stability was influenced by ambient O₂ concentrations as well as the local pH. GS-NO was also shown to be biologically active in this context, inducing in vitro relaxation of bronchial rings in a dose-dependent fashion.

Consideration of these data provide a possible means by which NO might exert important effects for a longer period of time than that possible considering the short half-life of free NO. It is conceivable, given this, that RS-NO compounds may be involved, at least in part, in the decrease in DLEC Na⁺ transport resulting from exposure to the supernatant of LPS-stimulated AM. This would represent a common mechanism underlying the qualitatively similar effects of supernatant treatment and coculture with endotoxin-stimulated AM. This, however, remains unproved.

An important role for ROS in this effect remains a possibility. Previous studies have provided somewhat conflicting results regarding the effect of oxidant exposure on epithelial ion transport. Matalon and colleagues, using ventral toad skin, demonstrated a significant decrease in total Iₑc resulting from addition of xanthine and xanthine oxidase (XO) to the apical (mucosal) side of the epithelial monolayer (155). This was associated with H₂O₂ generation and was completely inhibited by SOD and catalase treatment. No effect was seen when xanthine and XO were added to the serosal surface of the monolayer. It should be noted that, as this was intact tissue, it is uncertain if the basolateral surface of the epithelium was in contact with the generated
ROS. Direct addition of H$_2$O$_2$ to adult ATII cell monolayers also resulted in decreased $I_{sc}$ (128). This effect, however, was much more pronounced when the basolateral monolayer surface was treated, suggesting an inhibition of the Na$^+$-K$^+$-ATPase. In vivo experiments involving rats exposed to hyperoxia, have suggested an increase in epithelial ion transport resulting from oxidant stress. Nici and colleagues showed an increase in total lung mRNA levels for the Na$^+$-K$^+$-ATPase in rats exposed to >97% O$_2$ for 60 hours (182). Longer hyperoxic exposure was also shown to increase adult ATII cell expression of a protein recognized by an antibody raised against the biochemically purified Na$^+$ channel (98). This was associated with an increase in Na$^+$ transport as assessed by patch clamp studies. A more recent study demonstrated increased $\alpha$-rENaC levels and Na$^+$ transport resulting from similar hyperoxic exposure (281). Apart from highlighting the problems in comparing in vitro and in vivo studies, these data clearly demonstrate that the specific effects of oxidant exposure on epithelial Na$^+$ transport may vary.

Our studies involving NAC support an oxidant-based mechanism underlying the effect of activated AM on DLEC Na$^+$ transport. As outlined above, NAC can act as an antioxidant both by a direct radical scavenging effect (3) and through an increase in intracellular GSH levels. In contrast to NAC, no specific membrane transport mechanisms exist for DTT. This thiol agent is thought, therefore, to act as a radical scavenger largely outside the cell, without exerting a significant effect of intracellular GSH levels. In support of this, DTT treatment does not affect intracellular protein and non-protein thiol concentrations in DLEC (Dr. A.K. Tanswell, personal communication).
Given that DTT did not exert any modulatory effect on the AM-supernatant-induced impairment of DLEC Na\(^+\) transport, an extracellular scavenging action of NAC in this regard seems unlikely. It is possible, then, that the effect of NAC depends on its ability to support DLEC production of GSH. The specific role of GSH, however, as an intracellular and extracellular antioxidant or, perhaps, as a thiol substrate for other interactions, such as RS-NO formation, remains unknown.

The relevance of these studies to ALI will require additional future investigation. These results do suggest cellular mechanisms that may underly the clinical pulmonary derangements typical of ARDS. Matthay and colleagues have shown that a patient's ability to maintain alveolar fluid clearance is correlated to subsequent survival (158). The studies presented here suggest that mediators released from AM in the setting of ALI, particularly arising from an infectious insult, may impair the ability of lung epithelium to transport Na\(^+\), and therefore fluid, through a number of mechanisms. This includes effects even at the level of gene expression, profoundly influencing the most fundamental components of cell ion transport capability. A better understanding of these basic pathophysiologic processes will allow development of more rational, focused therapeutic strategies.
CONCLUSIONS AND DIRECTIONS FOR FUTURE RESEARCH

These studies have demonstrated that a soluble factor from LPS-stimulated rat AM can decrease fetal rat DLEC mRNA levels for α-, β-, and γ-rENaC, a specific lung epithelial Na⁺ channel. This potentially represents a previously unknown mechanism whereby activated inflammatory cells can contribute to lung injury, in addition to or in parallel with the impairment of functional DLEC Na⁺ transport, as reflected by a decrease in amiloride-sensitive $I_{sc}$ previously demonstrated in our laboratory. Furthermore, it is shown that the specific decrease in DLEC amiloride-sensitive $I_{sc}$ resulting from exposure to the supernatant of LPS-stimulated AM could be modulated by the thiol antioxidant NAC. This may provide further insight into the pathogenesis of ALI and suggest possible therapeutic strategies that might be investigated further.

Upon completion this thesis, several important questions remain unresolved. As outlined above, the specific AM product(s) responsible for these effects is unknown. We have shown that neither peroxynitrite nor NO appear to play a significant direct role. Investigating the potential action of AM-derived cytokines would seem to be a potentially fruitful next step. Studies examining this question might involve the use of specific inhibitors of various cytokines to determine if such agents could modulate the effect of LPS-stimulated AM on both DLEC rENaC expression and functional Na⁺ transport properties. In addition, combinations of various recognized, commercially available AM products could be added directly to cultured DLEC, and the effect on both rENaC gene expression, as well as ion transport properties as measured in the Ussing chamber, could be determined. As outlined above, such an approach has been utilized
in the study of the induction of lung epithelial iNOS expression (90). The role of ROS in this process could be more directly examined by determining the effect of direct oxidant exposure alone on DLEC rENaC mRNA expression.

In addition, the effect of the coculture model on DLEC rENaC protein expression has not been clarified. Specifically, it is not clear if reduced rENaC mRNA levels are associated with a similar decrease in channel protein levels within the cell or in the number of electrically active channels at the apical plasma membrane. Such analysis has been difficult in the past due to the comparatively low cellular rENaC protein levels as well as inconsistent results with rENaC antibodies required for western analyses or immunohistologic examination of tissues. As these issues are resolved, the effect of the clear changes in rENaC gene expression demonstrated in these studies on cellular levels of rENaC protein subunits and the channel protein itself, both quantitatively and temporally, can be studied. As indicated above, the nature of this relationship on a functional level, as reflected by cellular ion transport, will depend in large part on the half-life of the membrane protein, which also is yet to be clarified.

The potential importance of the in vitro alteration in Na⁺ channel gene expression to lung function in ALI would clearly be supported by demonstrating similar changes in vivo. Examination of whole lung rENaC mRNA levels in an in vivo model of ALI would allow perhaps a broader interpretation of the results of the studies presented here.

It has been speculated that the modulatory effect of NAC on the impairment of DLEC Na⁺ transport resulting from exposure to the cell-free supernatant of LPS-stimulated rat AM is related to effects on intracellular levels of GSH. This arises largely
from the observation that DTT, a thiol antioxidant that does not readily enter cells and therefore exerts a minimal effect on cellular GSH levels, failed to influence DLEC Na⁺ transport properties. Further studies are necessary, however, to more closely examine the specific role of fluctuations in GSH levels on DLEC ion transport. Specific inhibitors of GSH synthesis are available and could be used to directly study the effect of decreased availability of GSH on DLEC Na⁺ transport in the Ussing chamber.

The effect of NAC treatment in modulating the decrease in DLEC rENaC mRNA levels should also be evaluated. Similarly, interactions between NAC, GSH and NO in the observed effects of AM coculture on DLEC Iₑ represent a potentially interesting area of study, given the importance of RS-NO compounds in a variety of cellular functions, including ion transport. This might be approached initially by determining changes in S-nitrosoglutathione levels in the medium of DLEC cocultured with LPS-stimulated AM. Similar studies examining RS-NO levels in the supernatant of endotoxin-stimulated AM alone may lend support to the speculation that NO, shown to underly the effects of coculture on DLEC Na⁺ transport, also represents an important mediator of the similar effect exerted by AM supernatant.
REFERENCES


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