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Oxidant-Antioxidant Balance in Acute Lung Injury*

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ARDS is a disease process that is characterized by diffuse inflammation in the lung parenchyma. The involvement of inflammatory mediators in ARDS has been the subject of intense investigation, and oxidant-mediated tissue injury is likely to be important in the pathogenesis of ARDS. In response to various inflammatory stimuli, lung endothelial cells, alveolar cells, and airway epithelial cells, as well as activated alveolar macrophages, produce both nitric oxide and superoxide, which may react to form peroxynitrite, which can nitrate and oxidize key amino acids in various lung proteins, such as surfactant protein A, and inhibit their functions. The nitration and oxidation of a variety of crucial proteins present in the alveolar space have been shown to be associated with diminished function in vitro and also have been identified ex vivo in proteins sampled from patients with acute lung injury (ALI/ARDS). Various enzymes and low-molecular-weight scavengers that are present in the lung tissue and alveolar lining fluid decreased the concentration of these toxic species. The purpose of this brief chapter

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is to review the results from various studies demonstrating increased levels of reactive oxygen-nitrogen intermediates in the alveolar spaces of patients with ALI/ARDS.

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Key words: acute lung injury; alveolar epithelium; antioxidants; nitric oxide; nitrotyrosine; superoxide; surfactant protein A

Abbreviations: ALI = acute lung injury; BALF = BAL fluid; EF = edema fluid; GSH = glutathione; H$_2$O$_2$ = hydrogen peroxide; HPLC = high-pressure liquid chromatography; iNOS = inducible nitric oxide synthase; MDA = malondialdehyde; NO = nitric oxide; NOS = nitric oxide synthase; NOx = nitrate and nitrite; O$_2$ = superoxide; ONOO$^-$ = peroxynitrite; PI = proteinase inhibitor; RNs = reactive nitrogen species; ROS = reactive oxygen species; SP-A = surfactant protein A

Because of its unique structure and function, the lung becomes a vulnerable target during inflammation. The generation of oxidative and nitrosative species, which exert their effects both directly and indirectly, is a principal contributor to inflammatory injury. The terms oxidative and nitrosative refer to the formation of reactive oxygen species (ROS), such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals, and reactive nitrogen species (RNS), such as nitric oxide (NO), peroxynitrite (ONOO$^-$), and nitrogen dioxide. Many pathophysiological states of the lung lead to the up-regulation of inflammatory systems and involve enzyme systems to assist in the eradication of local inflammatory processes or to promote injury repair mechanisms. The failure to control inflammation locally can lead to the loss of control of these responses, with the end-result being systemic inflammatory organ dysfunction progressing to organ failure, and, in the most extreme circumstances, death.

Lung inflammatory-oxidant injury involves a complex cast of both humoral and cellular responses. A representative schema involves an insult such as infection and/or protracted shock inducing the expression of cytokines such as tumor necrosis factor-$\alpha$, which activates endothelial cells, epithelial cells, and macrophages, and recruits neutrophils, eosinophils, and other immune response cell lines. Neutrophils and other mononuclear cells require the participation of additional molecules to fully exert the inflammatory effect. These molecules include intracellular adhesion molecules (ICAM-1), selectins, and integrins (such as CD11/CD18). The activation and expression of these molecules allows for the adhesion, conformational change, and extravasation (emigration) of the neutrophil that may induce local injury and participate in the orchestration of systemic inflammation and all of its consequences.

Oxidants are generated as a result of the inflammatory response by phagocytic cells, such as mononuclear cells. In the presence of molecular oxygen and nitric oxide, nitrosoamid adenine dinucleotide phosphate oxidase, O$_2^-$ is produced via a one-electron reduction, with nitric oxide adenine dinucleotide phosphate donating the electron. The majority of O$_2^-$ will be dismutated by O$_2^-$ dismutase to form H$_2$O$_2$, which is central for bacterial eradication. Oxidants that are generated in excess of antioxidant defenses or that are lacking in antioxidant defenses can result in severe pulmonary inflammation leading to acute lung injury (ALI) or ARDS, as has been previously defined. Additionally, NO plays a multifaceted role in mediating inflammatory processes. Potential sources of NO in the lungs include activated AMs, neutrophils, alveolar type-II cells, endothelial cells, and airway cells. NO biosynthesis is enhanced during pulmonary inflammation via inducible NO synthase (iNOS) induction. iNOS has been immunolocalized to airway cells or human lung tissue that has been obtained from patients with ARDS, bacterial pneumonia, lung cancer, pulmonary sarcoidosis, idiopathic pulmonary fibrosis, and asthma. AMs isolated from the lungs of patients with tuberculosis or ARDS following sepsis have been shown to express iNOS. These findings raise the possibility that increased amounts of NO may be released during lung inflammation into the epithelial lining fluid, where it may have both beneficial (ie, antimicrobial) and detrimental (ie, tissue-damaging) effects.

Oxidants and Lung Injury

As mentioned before, the generation of oxidants during inflammatory conditions has been well-documented. The unequivocal measurement of ROS in biological systems is very difficult because the biological half-lives of the molecules are in the range of nanoseconds to milliseconds in length, coupled with the fact that the concentrations may vary dramatically within the time course of a particular disease state. However, measurements of stable by-products such as nitrate and nitrite, and modified proteins and lipids are performed routinely and often are used as indirect measures of oxidative stress.

Toxicity from oxygen metabolites released by stimulated neutrophils, macrophages, and other cells has been proposed as one of the significant mechanisms of lung injury. One of the initial studies published described the effects of inflammation on $\alpha_1$-proteinase inhibitor (PI), an inhibitor of elastase that is commonly found in BAL fluid (BALF). $\alpha_1$-PI was found to be inactive in the BAL fluid samples taken from patients with ARDS. This contrasted to the plasma samples containing $\alpha_1$-PI that were taken from the same patients, which were revealed to retain > 90% activity. This observation, coupled with the ability to restore the activity of $\alpha_1$-PI with the reducing agent, dithiothreitol, implicated the lung as the source of oxidation. Shortly thereafter, a different group measured expired fractions of H$_2$O$_2$, a more stable molecule that is a permeable and volatile oxidant. These samples were collected from patients with healthy lungs who were undergoing elective surgery (n = 13) and from critically ill patients with...
acute hypoxic respiratory failure (n = 55). The levels of expired breath condensates of H2O2 were observed to be significantly greater in patients with acute hypoxic respiratory failure and focal pulmonary infiltrates than in those without pulmonary infiltrates (mean [± SD], 2.34 ± 1.15 vs 0.99 ± 0.72 μmol/L, respectively), indirectly implicating increased oxidation. Interestingly, H2O2 concentrations were greatest in patients with head injuries and sepsis, whether pulmonary infiltrates were present or not. This unexpected finding suggested the participation of oxidants in sepsis and other forms of vital organ injury, such as trauma to the brain. Further studies have continued to create a solid foundation that implicates oxidant generation as a significant contributor to inflammatory-mediated lung injury. In fact, in one of the most recent studies, plasma hypoxanthine levels, a key cofactor that accumulates during intervals of hypoxia and leads to the production of O2 − and H2O2, were found to be significantly elevated in patients with ARDS. Moreover, the highest concentrations occurred in patients who did not survive (nonsurvivors, 37.48 ± 3.1 μM; survivors, 15.24 ± 2.09 μM; p < 0.001), implicating oxidative damage as an influential contributor to mortality.

There is now substantial experimental evidence that ROS and RNS may be involved in pulmonary epithelial injury in a variety of pathologic situations. The induction of immune complex alveolitis in rat lungs results in increased alveolar epithelial permeability, which is associated with the presence of elevated concentrations of NO decomposition products in BALF. Alveolar instillation of the NO synthase (NOS) inhibitor N(G)-monomethyl-L-arginine ameliorates NO production and alveolar epithelial injury. Similarly, paraglut-induced lung injury and ischemia-reperfusion-induced lung injury both are associated with the stimulation of NO synthesis and are abrogated by NOS inhibitors. Tracheal epithelial cytopathology induced by Bordetella pertussis is associated with the induction of NO synthesis and is markedly attenuated by the inhibition of NOS. Likewise, influenza virus-induced lung pathology in mice results from the increased expression of iNOS and the increased generation of NO. The administration of NOS inhibitors significantly improves the survival of influenza-infected mice. Additional evidence that RNS play a role in pulmonary inflammation is derived from studies utilizing transgenic Nos2−/− mice. Lung damage induced by the injection of lipopolysaccharide, influenza virus infection, or hemorrhage and resuscitation is markedly reduced in these mutant mice. Similarly, in an experimental murine model of allergic airway disease, the deletion of the Nos2 gene results in a significant decrease in eosinophil infiltration into the lungs.

**Increased Levels of NO in the BALF and Edema Fluid of Patients With ARDS**

ARDS is a disease process that is characterized by diffuse inflammation in the lung parenchyma. Concentrations of nitrate and nitrite (NOx), which are the stable breakdown by-products of NO, can be measured in biological fluids using the Greiss reaction. NOx concentrations were significantly higher than normal in the BALF from patients who were at risk for developing ARDS, as well as in the BALF of those with ARDS, and remained elevated throughout the course of ARDS. In all cases, the majority of the products detected were in the form of nitrate (> 90% nitrate and < 10% nitrite). NOx was in the BALF of healthy subjects (range, 2.5 to 4.3 μM; median, 2.5 μM). In patients who were at risk for ARDS, the NOx concentration in BALF from day 1 and day 3 after the onset of the risk factors (eg, multiple trauma, sepsis, or multiple transfusions) was significantly higher than in healthy subjects (Fig 1).

Patients who were at risk for ARDS and patients with established ARDS had similar concentrations of NOx in their BALF, either at the onset of ARDS or at any subsequent time. However, the patients studied on day 21 after the onset of ARDS, a time when the course of ARDS was waning, had the lowest concentrations of NOx in BALF. While there were no statistically significant differences in the BALF NOx concentrations from patients who were at risk for the development of ARDS, the NOx concentrations in the BALF of patients with ARDS who subsequently died were significantly higher on days 3 and 7.

The levels of NOx in the epithelial lining fluid of these patients cannot be easily estimated since they are diluted considerably (ie, > 50-fold) by the BALF. To address this issue, we measured NOx levels in the pulmonary edema fluid (EF) and plasma samples from patients with ALI/ARDS and, for comparison, in samples from patients with hydrostatic pulmonary edema. All of these patients were admitted to the ICUs at the University of California at San Francisco or San Francisco General Hospital between 1985 and 1998. Pulmonary EF was collected from each

![Figure 1. NOx in the BALF from healthy volunteers (NL), patients who were at risk for ARDS (Risk), and patients with established ARDS were studied at sequential times. The horizontal axis shows the patient group and the day on which the BAL was performed. The numbers in parentheses indicate the number of subjects in each group. The data are presented as box plots showing the 10th, 25th, 75th, and 90th percentiles and the median. * p ≤ 0.005 vs healthy subjects. Reprinted with permission from Sittipunt et al.](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21986/ on 04/10/2017)
patient within 30 min after endotracheal intubation by passing a standard 14F tracheal suction catheter through the endotracheal tube into a wedged position in a distal airway, as has been described previously.35 Pulmonary EF from patients with ALI had significantly higher levels of NOx compared to pulmonary EF from patients with hydrostatic pulmonary edema (mean [± SEM], 108 ± 13 vs 66 ± 9 μM, respectively; p < 0.05). In addition, patients with shock had higher mean plasma NOx levels than did those without shock (79 ± 11 vs 53 ± 12 μM, respectively; p < 0.05). The ratios of nitrate to nitrite in 11 EF samples and 9 plasma samples were 0.01 ± 0.005 vs 0.008 ± 0.004, respectively, indicating that > 90% of NOX was present as nitrate, which is in agreement with our BALF data (see above). Acidemia and increased anion gap, markers of systemic hypoperfusion, also were associated with twofold higher plasma NOx levels.

An additional benefit of sampling undiluted pulmonary EF rather than diluted BALF is the opportunity to measure alveolar fluid clearance by following serial changes in the protein concentration in the pulmonary EF. Patients with ALI who were able to concentrate alveolar protein (as a result of active sodium reabsorption) had a better prognosis than those that did not.35,36 Our results indicate that increased levels of NOx in EF samples were associated with slower rates of alveolar fluid clearance. One possible explanation for this finding is that the generation of ROS and RNS in the alveolar compartment leads to the nitration, oxidation, and inactivation of proteins that are important in alveolar epithelial sodium transport, such as the epithelial sodium channel. Indeed, intra-tracheal instillation of DETANONOnate, an NO donor, decreased amiloride-sensitive fluid clearance in rabbit lungs.37

EVIDENCE FOR THE EXISTENCE OF NITRATED PROTEINS IN VIVO

Several studies have provided evidence that nitration reactions occur in vitro during inflammatory processes. 3-Nitrotyrosine residues, which are products of the addition of a nitro-group (NO2) to the ortho position of the hydroxyl group of tyrosine, are stable end-products of RNS-mediated reactions. Therefore, they serve as footprints of RNS action, which are readily detectable by immunohistochemistry, enzyme-linked immunosorbent assay or high-pressure liquid chromatography (HPLC).38 Nitrotyrosine is commonly detected in tissues that have been infiltrated by neutrophils and monocytes during infections and inflammatory processes.15,39 In vitro, proteins can be nitrated either by ONOO− or by reactive intermediates that have been generated by the myeloperoxidase-catalyzed reaction of reactive species released from activated neutrophils.40,41 Immunohistochemical studies showing evidence of nitrotyrosine residue formation on proteins in cells taken from the lung tissues of pediatric patients with ALI were first reported by Haddad et al.42 Oxidant-mediated tissue injury is likely to be important in the pathogenesis of ARDS.3,39

Protein nitration and oxidation by ROS and RNS in vitro have been associated with the diminished function of a variety of crucial proteins present in the alveolar space, including α1-PI and surfactant protein A (SP-A).42,43 Gole et al44 reported the presence of nitrated ceruloplasmin, transferrin, α1-protease inhibitor, α1-anti-chymotrypsin, and β-chain fibrinogen in the plasma of patients with ALI/ARDS. Using quantitative enzyme-linked immunosorbent assay and HPLC, we detected significant levels of protein-associated nitrotyrosine (approximately 400 to 500 pmol/mg protein) in EF samples from both ALI/ARDS and patients with ARDS.15 These levels of nitrotyrosine are at least one order of magnitude higher than those found in normal human BALF (28 pmol/mg protein),45 normal rat lung tissue (approximately 30 pmol/mg protein),46 normal human serum albumin (approximately 30 pmol/mg protein),47 or normal human plasma low-density lipoprotein (approximately 85 pmol/mg protein).48 Lamb et al49 also measured nitrotyrosine content in the BALF of patients with severe ARDS and in healthy volunteers using HPLC, although their values were considerably higher than those reported by Sittipunt et al45 and Zhu et al.44

Nitrated pulmonary SP-A also was detected in the EF but not in the plasma of patients with ALI after immuno-precipitation with a specific antibody against this protein (Fig 2). Although we previously demonstrated that SP-A is nitrated and oxidized in vitro, using lipopolysaccharide-stimulated rat AMs as the source of the reactive species,49 this is the first in vitro evidence for the nitration of a specific protein in the alveolar spaces of the human lung. The results of previous in vitro studies indicated that nitrated SP-A loses its ability to enhance the adherence of Pneumocystis carinii to rat AMs45 and inhibits the killing of Mycoplasma pulmonis by mouse AMs.46 (Hickman-Davis et al, unpublished observations). Also, the nitration of human SP-A by ONOO− or tetranitromethane inhibited its lipid aggregation and mannose-binding activities.50 Finally, SP-A isolated from the lungs of lambs that had been exposed to high concentrations of inhaled NO had a decreased ability to aggregate lipids. Thus, the nitration of SP-A may be one of the factors responsible for the increased susceptibility of patients with ARDS to nosocomial infections.51 Interestingly, despite being present at high concentrations in the epithelial lining fluid of patients with ARDS, albumin was nitrated to a much lesser degree than was SP-A.52

OXIDANT-ANTIOXIDANT BALANCE IN ARDS

While the direct measurements of oxidants poses problems, the monitoring of antioxidant concentrations and/or oxidant-antioxidant balance also has been assessed. For instance, selected antioxidants were measured including plasma ascorbate, a major plasma antioxidant, and were significantly decreased in patients with ongoing ARDS when compared to healthy control subjects.52 In addition, ubiquinol, a key lipid-soluble antioxidant residing in the membranes of the mitochondria, was significantly decreased in patients with ARDS as well. Interestingly, the levels of α-tocopherol, an additional plasma antioxidant, were unchanged when the two groups were compared. In a series of separate experiments, after plasma from a healthy donor was incubated with activated polymorpho-
contrast, levels of catalase, which is a scavenger of $H_2O_2$, compared to samples from healthy control subjects. In GSH, and levels of GSH were found to be decreased when patients with ARDS were analyzed for the presence of conditions did or did not eventually progress to ARDS. Additional studies have confirmed that, in the pro-oxidant pulmonary milieu observed in patients with sepsis and lung injury, antioxidant responses are significantly elevated when compared to those of control patients.

Describing the influences of inflammation on host oxidant production and antioxidant defense mechanisms have been extremely important in our understanding of ALI/ARDS. However, treatment strategies have fallen short secondary to their simplicity. As well, there is a paucity of data in this area. Eight patients with ARDS receiving “standardized” total parenteral nutrition were investigated and compared to 17 healthy individuals who served as control subjects in an attempt to assess the influence of micronutrients on the oxidative system. The measurements of plasma antioxidants and antioxidant enzyme systems were measured at baseline and on days 3 and 6, and were compared to those of control subjects who were receiving standard diets without vitamin or trace element supplementation. In addition, the lipid peroxidation product, malondialdehyde (MDA), $O_2^-$ anion, and $H_2O_2$ were measured over the same time points. Plasma levels of $\alpha$-tocopherol, ascorbate, $\beta$-carotene, and selenium were reduced when compared to those of control subjects. MDA was significantly increased and was observed to increase significantly over the 6-day interval. The authors concluded that in patients with ARDS, the antioxidative system is severely compromised and there is evidence of progressive oxidant stress, as per the steady increase in MDA. Thus, the administration of standardized total parenteral nutrition seems inadequate to compensate for the increased requirements. In a contrasting study, when patients with ARDS were entered into a prospective, multicentered, double-blind, randomized control trial comparing a specialized enteral formulation containing fish, borage oil, and elevated antioxidants vs an isonitrogenous, isocaloric standard diet, beneficial anti-inflammatory effects were observed that translated into reductions in the number of days spent receiving mechanical ventilation, in the lengths of stay in the ICU, and in instances of new organ failure.

How does the above information translate to the bedside care of the patient who has experienced a lung injury? The data presented herein indicate that stable decomposition products of both NO and intermediates generated by its reaction with ROS are detected in high
concentrations in both the BALF and EF of patients who are at risk of developing ARDS or who have established ARDS. Levels of reactive species correlate both with the outcome of the disease and the severity of the injury to the alveolar epithelium. Finally, significant levels of nitrated SP-A and fibrinogen are detected in the EF and plasma of patients with ARDS. Although the findings of in vitro studies indicate that the nitration of both proteins leads to diminished functioning, it still needs to be established whether there are sufficient levels of nitrination in vivo to contribute to the pathogenesis of ARDS. Finally, these findings indicate that antioxidant enzymes and scavengers of reactive oxygen nitrogen intermediates may be useful in decreasing the severity of ARDS. Clearly, additional studies in this area of research are needed.

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Goblet Cell and Mucin Gene Abnormalities in Asthma*

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Goblet cell hyperplasia (GCH) has been established as a pathologic characteristic of mild, moderate, and severe asthma. Abnormalities in goblet cell number are accomplished by changes in stored and secreted mucin (MUC). The functional consequences of these changes in MUC stores and secretion can contribute to the pathophysiology of multiple clinical abnormalities in patients with asthma, including sputum production, airway narrowing, exacerbations, and accelerated loss in lung function. CD4+ T cells and their T-helper type-2 cytokine products are important mediators of GCH, and MUC5AC is the dominant MUC gene that is expressed in goblet cells. The mechanism of cytokine-induced GCH, the relationships between MUC gene up-regulation and GCH, and the role of ion channels are all currently being explored. The process of working out the molecular mechanisms of GCH and goblet cell degranulation should provide new targets for novel therapeutic interventions. Such new treatments are urgently needed, because mucous hypersecretion is an important cause of morbidity and mortality in patients with asthma, and no specific treatments are available.

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Key words: asthma; goblet cells; mucin genes; mucin glycoproteins

Abbreviations: AB = alcian blue; EGFR = epidermal growth factor receptor; GCH = goblet cell hyperplasia; IL = interleu

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