Lung Injury and Oxidoreductases

J.R. Hoidal, P. Xu, T. Huecksteadt, K.A. Sanders, K. Pfeffer, and A.B. Sturrock
Department of Internal Medicine, University of Utah Health Sciences Center, Salt Lake City, Utah

Acute lung injury represents a wide spectrum of pathologic processes, the most severe end of the spectrum being the acute respiratory distress syndrome. Reactive oxygen intermediates have been implicated as important in the pathobiology of acute lung injury. The endogenous sources that contribute to the generation of reactive oxygen intermediates in acute lung injury are poorly defined but probably include the molybdenum hydroxylases, NAD(P)H oxidoreductases, the mitochondrial electron transport chain, and arachidonic acid-metabolizing enzymes. Our laboratory has focused, in particular, on the regulation of two of these enzyme systems, xanthine oxidoreductase (XDH/XO) and NAD(P)H oxidase. We observe that gene expression of XDH/XO is regulated in a cell-specific manner and is markedly affected by inflammatory cytokines, steroids, and physiologic events such as hypoxia. Posttranslational processing is also important in regulating XDH/XO activity. More recently, the laboratory has characterized an NAD(P)H oxidase in vascular cells. The cytochrome components of the oxidase, gp91 and p22, appear similar to the components present in phagocytic cells that contribute to their respiratory burst. In human vascular endothelial and smooth muscle cells, oncostatin M potently induces gp91 expression. We believe that regulation of gp91 is a central controlling factor in expression of the vascular NAD(P)H oxidase. In summary, the studies support the concept that the oxidoreductases of vascular cells are expressed in a highly regulated and self-specific fashion. — Environ Health Perspect 106(Suppl 5):1235–1239 (1998). http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-5/1235-1239hoidal/abstract.html

Key words: lung injury, xanthine oxidoreductase, NAD(P)H oxidase, gene regulation

The Clinical Problem

Acute lung injury contributes to the morbidity and mortality of patients throughout the world at tremendous human and financial costs. It is a syndrome that includes pulmonary vasoconstriction, inflammation, and increased permeability of both the alveolar capillary endothelium and epithelium, resulting in arterial hypoxemia resistant to oxygen therapy and diffuse infiltrates on chest X-ray. This injury is associated most often with sepsis, aspiration, pneumonia, massive trauma, or ischemia–reperfusion injury and is frequently the initial and overriding component of the multiple organ dysfunction syndrome. Acute lung injury represents a wide spectrum of pathologic processes, the most severe end of the spectrum being the acute respiratory distress syndrome (ARDS). Widely reported figures place the incidence of ARDS at approximately 150,000 cases per year in the United States, although the figure is challenged and likely an overestimate (1,2). The mortality from ARDS is high, ranging from 30 to 90% in reported studies [reviewed by Bernard et al. (2)]. Because of this excessive morbidity and mortality, a better understanding of the pathobiology of acute lung injury is necessary to design effective therapies or prophylactic measures to decrease the incidence and severity of this devastating disorder.

Oxidants and Acute Lung Injury

A number of independent pathogenic mechanisms may cause acute lung injury. One group of inorganic compounds with a high degree of chemical reactivity that has been implicated is the reactive oxygen intermediates (ROI), including superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical, and products of myeloperoxidase. ARDS patients have increased production of oxidants in their lungs, as indicated by an increased concentration of H_2O_2 in expired air (3), and a deficiency in alveolar epithelial lining fluid glutathione with a greater percentage in the oxidized form (4). In addition, some of the detrimental effects of ROI are mediated through the reaction of O_2^- with nitric oxide to form the potent oxidant ONOO^- (5). Potentially toxic levels of ONOO^- are present in the lungs of ARDS patients (5).

Animal models provide strong support for the critical involvement of oxidants in acute lung injury. The role has been demonstrated in models of lung injury from sepsis (6), viral pneumonia (7), ischemia–reperfusion (8), hemorrhagic shock, a systemic ischemia–reperfusion insult (9), and hyperoxia (10). Antioxidant enzymes or scavengers markedly reduce the lung injury in these models.

Several mechanisms have been identified by which oxidants can cause lung dysfunction. Oxidants react with many cellular components, oxidizing proteins, lipids, DNA bases, enzymes for intermediary metabolism, and extracellular matrix components including collagen and hyaluronic acid (11–13). The production of PG12, isoprostanes, and lipoxygenase products is altered, causing defects in platelet aggregation and vasorelaxation (14,15). Depending on the extent of oxidant stress, cells exposed to ROI will undergo apoptosis or necrosis (16). In endothelial and epithelial cells, oxidant injury may also impair macromolecular barrier function, which leads to pulmonary edema (17). As one possible mechanism, O_2^- in posthypoxic endothelial cells may directly affect the actin cytoskeleton by changing the redox state of actin regulatory proteins or of actin.
itself, thereby inducing actin polymer formation (18). Finally, $H_2O_2$ and $O_2^-$ can initiate production of potent chemotaxins (19) or can increase leukocyte adhesion to endothelium via activation of nuclear factor-kappa B-mediated transcription of integrin genes (20,21). Thus, localized production of ROI may initiate a cascade that culminates in a fulminating inflammatory response, tissue destruction, and organ malfunction.

**Source of Oxidants**

Although evidence supporting a role for oxidants in the pathobiology of acute lung injury is substantial, key questions that still need to be addressed include: a) What are the endogenous sources of ROI that initiate the lung injury, and b) how are they regulated? The endogenous sources that contribute to the generation of ROI in the lung are poorly defined but likely include the molybdenum hydroxylases (xanthine dehydrogenase/oxidase [XDH/XO] and aldehyde oxidase), NADPH or NADPH oxidoreductases (including the phagocytic cell oxidase cytochrome P450 and nitric oxide synthase), the mitochondrial electron transport chain, and arachidonic acid-metabolizing enzymes such as cyclooxygenase. In the lung, the best studied sources for ROI are XDH/XO and the NAD(P)H oxidase of phagocytic cells. During the past 5 years our laboratory has concentrated on XDH/XO and more recently on an incompletely described NAD(P)H oxidase present in vascular cells. (Whether NADH or NADPH is the preferred substrate is not known.)

**Xanthine Oxidoreductase and Its Regulation**

XDH, an NAD-dependent dehydrogenase, catalyzes the final two reactions of purine catabolism and as such is the rate-limiting enzyme in their oxidative metabolism. The enzyme is a homo-dimer with a subunit $M_f$ of about 150,000 daltons. Each subunit contains four redox active centers: two iron sulfur, one FAD, and one molybdopterin. In mammalian species, XDH can be readily converted to XO by reversible sulfhydryl oxidation or by irreversible posttranslational modification. The oxidation by XDH of hypoxanthine to xanthine and the subsequent oxidation of xanthine generates the powerful antioxidant urate, which directly scavenges ROI and chelates iron (22). Ames and colleagues (22) proposed that one mechanism for the lengthening life-span and the decrease in age-specific cancer rates during primate evolution may be attributed to the high plasma levels of urate that are 10-fold greater than in lower mammals.

Juxtaposed to the potentially protective physiologic function of XDH in providing urate as an antioxidant defense is the ability of XO to generate $O_2^-$ and $H_2O_2$ from molecular oxygen. Originally proposed as mediators of ischemia–reperfusion injury (8), XO-derived ROI have now been implicated in the pathogenesis of cellular injury in a wide variety of pulmonary disorders including viral pneumonia (23), and following sepsis (24) or hemorrhagic shock (25).

Sources of XDH/XO that mediate injury include resident lung cells such as endothelial and epithelial cells, both important sources of the enzyme in some species (26,27). Additionally, diverse pathologic processes result in circulating XDH/XO. XDH/XO released from tissues into plasma can bind to sulfated glycosaminoglycans on vascular endothelial cells to produce site-specific oxidant injury to organs remote from the site of release (28). As one example, XDH/XO-rich effluent from reperfused ischemic liver accumulates in the lung in an enzymatically active form (29). Importantly, patients with ARDS (3), viral infections (30), or tumor–related ischemia–reperfusion injury have markedly increased levels of plasma XDH/XO (31). These observations provide a possible explanation for lung injury following a peripheral insult.

Most studies investigating the role of XDH/XO in disease pathogenesis have applied the axiom that the significance of XDH/XO as a mechanism for generation of ROI in tissues is determined simply by the rate of conversion of XDH to XO. However, recent molecular and biochemical approaches have provided important new insights into the regulation of XDH/XO that may apply not only to ischemia–reperfusion but also to inflammatory disorders. Thus, besides the simple conversion of XDH to XO, additional mechanisms may regulate the enzyme leading to enhanced ROI generation.

Characterization of the cDNA for XDH/XO by Amaya (32) has made possible the evaluation of the transcriptional regulation of XDH/XO. Our laboratory first demonstrated transcriptional regulation of the enzyme and found that interferon gamma (IFN-γ) selectively induced gene activation in pulmonary microvascular cells and in rat lungs in vivo (26). We subsequently demonstrated transcriptional regulation of XDH/XO in epithelial cells by cytokines and steroids in a pattern analogous to the profile seen by acute phase reactants in response to injury, trauma, or infection (33). Although the acute phase response is thought to protect the host, the role of XDH/XO upregulation as part of this reaction could be deleterious because of the increased generation of ROI. More recently, transcriptional activation of XDH/XO has been shown in response to hypoxia (34) or after lipopolysaccharide (LPS) exposure in vivo (35). The importance of these studies rests on their demonstration that XDH/XO gene expression is regulated in a cell-specific manner and is markedly affected by inflammatory cytokines, steroids, and physiologic events such as hypoxia.

A second important advance is the information about posttranscriptional and posttranslational regulation of XDH/XO. We have demonstrated recently that hypoxia not only transcriptionally regulates XDH/XO, as suggested by Hassoun and colleagues (34), but also enhances enzyme activity by as yet undefined posttranslational processes that do not involve conversion of XDH to XO (36). Posttranscriptional regulation by iron (37) and posttranslational regulation by nitric oxide (38) have also been established. Moreover, the recent evidence that human XDH/XO can undergo activation–deactivation cycles at the molybdenum redox center (39) further emphasizes the potential importance of posttranslational regulation of XDH/XO.

A third important advance is the evidence that XDH/XO acts not only on purines but also on reducing substrates including NADH with resultant $O_2^-$ formation. Recent studies indicate that XDH/XO can oxidize NADH, producing $O_2^-$ at 0.23 μmol/min/mg (40). These findings suggest an alternative mechanism for ischemia–reperfusion injury. By this alternative mechanism, ischemia elevates intracellular NADH levels, which leads to $O_2^-$ generation by XDH/XO on reoxygenation of tissues. This mechanism does not depend on XDH-to-XO conversion (a controversial issue). We believe that the NADH oxidation does not involve the molybdenum center of the enzyme (Figure 1) and therefore would not be inhibited by purine analogs such as allopurinol or oxypurinol, or by a supplementation with taurine. This observation is noteworthy.
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In light of the recent demonstration of an uncharacterized NADH oxidase as an important source of ROI in lung cells and reperfusion injury (41,42) and the striking increase in cellular NADH occurring during ischemia.

In summary, recent information indicates that XDH/XO is regulated by both transcriptional and posttranslational events possibly acting in concert to increase the enzyme's ability to generate ROI, and that its ability to generate ROI may not depend solely on the half-oxidative reaction occurring at the molybdenum redox center.

**NAD(P)H Oxidase in Vascular Cells**

Knowledge about the structure and activation of NAD(P)H oxidase comes mostly from studies in neutrophils in which NAD(P)H is responsible for the respiratory burst essential to the microbicidal activity of these cells. NAD(P)H oxidase is a multisubunit complex that generates O$_2^-$ in one-electron reduction of O$_2$ using electrons supplied by NAD(P)H [reviewed by Dinauer (43)]. The oxidase is expressed at high levels in phagocytes, where O$_2^-$ is the precursor to H$_2$O$_2$ and to other reactive oxidants used to kill bacteria and fungi. The oxidase consists of two membrane proteins, gp91 and p22, that together form a unique cytochrome with a redox midpoint potential of ~245 mV and a reduced minus oxidized difference spectrum of 558. Based on studies of subjects with chronic granulomatous disease (CGD), at least two cytosolic peptides (p47 and p67) are also essential. Several other cytosolic components that appear to participate in the activity of the phagocyte NAD(P)H oxidase have been identified and include a small G protein (known as Rac2 in humans), rho-GDI, and p40$^{map}$. In neutrophils, oxidase is activated by assembly of the cytosolic proteins with the membrane components. Heritable defects of either gp91, p22, p47, or p67 are the basis for CGD, a disorder of white cell function characterized by recurrent, severe, bacterial and fungal infections. The genes encoding the different phagocyte NAD(P)H oxidase components have been cloned and respective cDNA probes generated, thus allowing studies on the expression and localization of different mRNA transcripts in cells and tissues.

During the past few years, evidence has been accumulating that a similar enzyme complex is present and exerts a variety of functions in nonphagocytic cells. Both the endothelium and vascular smooth muscle contain a membrane-bound oxidase that uses NADH and NADPH as substrates for electron transfer to O$_2$ that appears similar to the NAD(P)H oxidase of neutrophils (44-46). In cultured vascular smooth muscle cells, the oxidase is a significant source of O$_2^-$ formation (47). For example, in calf pulmonary and coronary artery smooth muscle, this oxidase accounts for the majority of the O$_2^-$ generated (45,48). Importantly, it uses a cytochrome b$_{558}$ electron transport system (45). There are, however, important differences between the vascular oxidase and the neutrophil oxidase. These include the delayed time course for activation, low output, and in some studies, the preference for NADH rather than NADPH of the vascular oxidase. The low-output property does not detract from the importance of the vascular oxidase as an initiator of endothelial or smooth muscle dysfunction, and it may function as a signaling system for gene activation (49).

To date, there has not been a comprehensive molecular characterization of the vascular NAD(P)H oxidase. Ushio-Fukai and co-workers (50) have provided evidence that p22 is a critical component of the O$_2^-$ generating vascular NAD(P)H oxidase and suggest a central role for this oxidase system in vascular hypertrophy. However, it is unlikely that p22 serves as a complete oxidase on its own, as it can function only as a one-electron acceptor and lacks substrate-binding sites and flavin-binding sites (43). The other subunit of the electron transport element of the vascular NAD(P)H oxidase has been more elusive. Recent immunohistochemical studies have suggested expression of gp91 in vascular smooth muscle cells (46), but this has not been a consistent finding (51). Because the substrate specificity of the neutrophil NAD(P)H oxidase resides in gp91, it is likely that it is this subunit that determines the unique properties of the vascular oxidase.

To define the components responsible for the NAD(P)H oxidase activity in human vascular cells, we screened a human umbilical vein endothelial cell (HUVEC) cDNA library (in 1gt1; Clontech, Palo Alto, CA) using probes for gp91, p22, p47, and p67. The probes were generated by polymerase chain reaction using primers designed from phagocyte sequences. To date we have isolated from the HUVEC library a full-length p22 cDNA, a 1200-bp fragment of gp91, and a 500-bp fragment of p67. We have also amplified by reverse transcriptase-polymerase chain reaction (RT–PCR) a 350-bp fragment from HUVEC mRNA using primers specific for p47 but have not yet isolated a p47 clone by library screening. These fragments have been sequenced and are identical to their neutrophil counterparts. Likewise, in fragments derived from pulmonary artery smooth muscle cells (PASMC) by RT-PCR, we have sequenced approximately 300 bp from the translated region of p22, p47, and p67, and, as in the case of endothelial cells, the sequences are identical to their phagocytic counterparts.

To initiate studies to define the factors that determine cell-specific gene expression, we used RT–PCR to identify transcripts for the NAD(P)H oxidase components in isolated endothelial and vascular smooth muscle cells. We have consistently found transcripts for p22, p47, and p67 in HUVEC. Transcripts for gp91 were usually not detected or were detected at low levels in HUVEC in the absence of an agonist. Transcripts for p22 were also detected in human aortic and lung microvascular endothelial cells, and intestinal and PASMC. Using Northern blot analysis, we detected p22 but not gp91 in HUVEC and PASMC. Our interpretation is that under basal conditions, p22 transcripts are expressed in substantially greater amounts than gp91 transcripts in HUVEC and PASMC.

Recently, we have initiated studies to evaluate factors that may regulate the expression of the NAD(P)H oxidase components. We focused on the effects of cytokines and hypoxia because of their relevance to ARDS. Addition of LPS, tumor necrosis factor α, IFN-γ, or interleukin-1β

![Figure 1. Electron transfer pathways within XDH. XDH as an NADH oxidase. Mo molybdenum; FeS iron(II) sulfide.](image-url)
neither induced gp91 gene expression in HUVEC or PASM C nor changed the level of mRNA transcripts for the other three components of the oxidase (OSM) potently induced gp91 in HUVEC and PASM C. The induction of gp91 required 8- to 24-hr exposure to OSM and was blocked by addition of cycloheximide (5 μg/ml), suggesting a requirement for intermediate protein synthesis. At variance with the induction of mRNA transcripts for gp91 by OSM, there was no induction of the other NAD(P)H oxidase components by any of the cytokines. This suggests that whereas gp91 is inducible in HUVEC and PASM C, p22, p47, and p67 are constitutively expressed in these cells. On the basis of these findings, we propose that regulation of gp91 is a central controlling factor in the expression of the vascular NAD(P)H oxidase.

In summary, recent studies support the concept that the oxidoreductases of vascular cells are expressed in a highly regulated and cell-specific fashion. The regulation of these enzyme systems is likely to have a profound impact on their role in acute lung injury.

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