Lung redox homeostasis: emerging concepts

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Merker, Marilyn P., Bruce R. Pitt, Augustine M. Choi, Paul M. Hassoun, Christopher A. Dawson, and Aron B. Fisher. Lung redox homeostasis: emerging concepts. Am J Physiol Lung Cell Mol Physiol 279: L413–L417, 2000.—This symposium was organized to present some aspects of current research pertaining to lung redox function. Focuses of the symposium were on roles of pulmonary endothelial NADPH oxidase, xanthine oxidase (XO)/xanthine dehydrogenase (XDH), heme oxygenase (HO), transplasma membrane electron transport (TPMET), and the zinc binding protein metallothionein (MT) in the propagation and/or protection of the lung or other organs from oxidative injury. The presentations were chosen to reflect the roles of both intracellular (metallothionein, XO/XDH, and HO) and plasma membrane (NADPH oxidase, XO/XDH, and unidentified TPMET) redox proteins in these processes. Although the lung endothelium was the predominant cell type under consideration, at least some of the proposed mechanisms operate in or affect other cell types and organs as well.

endothelium; oxidation-reduction; oxidative stress

EFFECT OF NITRIC OXIDE ON METAL ION HOMEOSTASIS IN PULMONARY ENDOTHELIAL CELLS

The profound effects of nitric oxide (NO) on cellular metabolism are in part related to its affinity for heme and nonheme iron, including targets such as the Fe-S clusters of aconitase/iron response element binding protein, hemoproteins such as catalase or cyclooxygenase 1, and heme oxygenase (HO)-1 (8, 24). After iron, zinc is the major intracellular metal. Indeed, 1–10% of the genome of species from invertebrates to humans are zinc proteins, including transcription factors, enzymes, ion channels, and structural proteins. Zinc is redox inert, but by binding to cysteine, it creates functionally important protein folds protecting the target molecule from oxidation. Such biophysical effects contribute to the role of zinc in gene expression, DNA synthesis, enzymatic catalysis, hormonal storage, neurotransmission, immunology, and cell injury.

Metallothionein (MT) is an intracellular cysteine-rich (30 mol/100 ml) metal binding protein and a
critical component of zinc homeostasis. It serves a unique role, linking changes in free intracellular zinc to altered redox status of cells. In this regard, in vitro data show that NO can S-nitrosylate MT and that NO can increase labile zinc in aortic endothelial cells. Dr. Pitt summarized recent progress from his laboratory demonstrating that S-nitrosylation of MT links NO to zinc homeostasis in pulmonary endothelial cells.

To directly study the interaction between MT and NO in live cells, a new fusion protein consisting of MT sandwiched between two mutant green fluorescent proteins (GFPs) was prepared (21). In vitro studies with this chimera (FRET-MT) demonstrated that fluorescent resonance energy transfer (FRET) can be used to follow conformational changes indicative of metal release from MT. Imaging experiments with live pulmonary endothelial cells showed that agents that increase cytoplasmic Ca²⁺ (the Ca²⁺ ionophore A-23187, carbachol, and bradykinin) act via endogenously generated NO to rapidly and persistently release metal from MT, an effect that can be mimicked by NO donors such as S-nitrosoglutathione or exogenous NO gas.

Additional microspectrofluorometric studies with the zinc-sensitive fluorophore Zinquin revealed that exposure of pulmonary endothelial cells to the NO donor S-nitrosocysteine resulted in a rapid increase in free zinc that was mimicked by increasing intracellular zinc (50 μM) or by the zinc ionophore pyrithione (21). The NO- and zinc-induced changes were sensitive to the zinc chelator N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), further demonstrating that Zinquin was reporting zinc.

Exposure of pulmonary endothelial cells to the NO donor S-nitroso-N-acetylpenicillamine or virus-mediated direct gene transfer of inducible nitric oxide synthase (iNOS) reduced the sensitivity of these cells to lipopolysaccharide (LPS)-induced apoptosis (6). This NO-induced resistance is associated with the inhibition of LPS-induced activation of caspase-3, an element in numerous apoptotic pathways and itself a potential zinc-inhibitable protease. Preliminary experiments indicating reversal of the NO-induced resistance by simultaneous exposure of the cells to the zinc chelator TPEN suggest the possibility that the well-known antiapoptotic effects of zinc (perhaps via caspase-3 activation) play a role in the NO-mediated resistance to LPS toxicity in pulmonary endothelial cells in culture.

Future studies will require more quantitative measurements of labile zinc and new fluorophores with isobestic spectral properties and higher affinities for free zinc than Zinquin. In addition to MT, other components of intracellular zinc homeostasis, including members of the zinc transport protein gene family, need to be investigated in pulmonary endothelial cells for a fuller understanding of the role of zinc in endothelial cell function in health and disease.

To evaluate the capability of pulmonary endothelium to carry out TPMET-mediated reduction of extracellular electron acceptors, a capillary- and plasma membrane-impermeant thiazine electron acceptor, toluidine blue O polyacrylamide polymer (TBOP), was synthesized (5). TBOP was reduced by endothelial cells both in culture and in the perfused lung. All of the TBOP was recovered in the extracellular medium or lung perfusate, indicating that neither the oxidized nor reduced forms of TBOP entered cells, and various control studies appeared to rule out release of a TBOP reducing agent from the cells or lung into the medium. The observations indicate that endothelial cells have the capability of altering the redox status of at least some bloodborne redox active substances that are confined to the blood.

The fact that thiazine reductases are commonly also quinone reductases led to speculation that natural endothelial TPMET electron acceptors might include spent quinoid antioxidants. This speculation was bolstered by the observation that when endothelial cells were incubated with certain quinones, such as duroquinone, the reduced hydroquinone form appeared in the extracellular medium. Although the study did not reveal whether the reduction occurred at the cell surface, it did demonstrate that the endothelium can modify the redox composition of the extracellular medium. To evaluate the possibility that TPMET might have contributed to the appearance of the hydroquinones in the medium, quinone reduction by endothelial cell plasma membrane preparations was studied. Luminally accessible endothelial plasma membrane proteins were labeled in the perfused lung by single-pass bolus injection of the cell membrane-impermeant amine reactive reagent sulfo succinimidyl-6-(biotinamido)hexanoate (sulfo-NHS-LC-biotin) (10). To separate the plasma membrane proteins labeled with biotin from other cell proteins, the lungs were homog-

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2 Presented by Marilyn P. Merker.
enized, and the biotin-labeled proteins in the lung homogenate were captured on avidin-coated beads. When the beads, now coated with the cell surface proteins, were mixed with the quinone form of coenzyme Q₁₀ and the electron donor NAD(P)H, the beads mediated a diphenyleneiodonium-sensitive reduction of quinone.

These studies demonstrate that some isolated endothelial cell surface plasma membrane proteins are capable of mediating quinone reduction, consistent with the hypothesis that pulmonary endothelial TPMET may regenerate antioxidant hydroquinones from spent, oxidized quinones present in the extracellular medium. This redox activity of the endothelial surface is also likely to be involved in modifying the redox status of other bloodborne substances such as redox active metals, toxins, and antibiotics.

**EMERGING ROLE OF CARBON MONOXIDE IN LUNG BIOLOGY AND PATHOLOGY**

HO catalyzes the rate-limiting step in the degradation of heme to yield equimolar quantities of biliverdin IXa, carbon monoxide (CO), and iron (7). Three isoforms of HO exist: HO-1 is the inducible isoform, whereas HO-2 and HO-3 are constitutively expressed (7). Heme, the major substrate of HO-1, as well as heavy metals, cytokines, endotoxin, and various agents that induce oxidative stress all induce HO-1 expression (7). The diversity of agents that induce HO-1 has led to the speculation that it may play a vital role in cellular homeostasis. Recent analyses of HO-1-null mice and a HO-1-deficient human have strengthened the emerging paradigm that HO-1 is indeed an important molecule in host defense against oxidant stress, but the mechanism(s) by which HO-1 provides protection is poorly understood. Based on the observations that endogenous induction of HO-1 provides protection against oxidative stress (7, 19), the hypothesis that the gaseous molecule CO, a major by-product of heme catalysis by HO, mediates the protection was pursued.

Animals exposed to a low concentration of CO (50–500 parts/million) exhibited a marked tolerance to lethal hyperoxia in vivo; survival was associated with significant attenuation of hyperoxia-induced lung injury (decrease in volume of pleural effusion, protein accumulation in the airways, and neutrophil influx into the airways) (20). In rats exposed to hyperoxia in the presence of a low concentration of CO, histological examination showed that the lungs were devoid of airway and parenchymal inflammation, fibrin deposition, and pulmonary edema. Furthermore, in rats in which endogenous HO enzyme activity was inhibited with the HO inhibitor tin protoporphyrin, exogenous CO protected against hyperoxia-induced lung injury. Low concentrations of CO also had potent anti-inflammatory effects in vivo and in vitro, including inhibition of expression of LPS-induced proinflammatory cytokines, including tumor necrosis factor-α, interleukin (IL)-1β, and macrophage inflammatory protein-1β, and augmentation of LPS-induced expression of the anti-inflammatory cytokine IL-10 (18). These anti-inflammatory effects of CO were mediated via a mitogen-activated protein kinase pathway and not a guanylyl cyclase-cGMP- or nitric oxide-dependent pathway. These data suggest that CO may play an important protective role in other oxidant-induced tissue injury and inflammatory states.

**REGULATION OF XANTHINE OXIDASE AND ROLE IN OXIDATIVE LUNG INJURY**

Through its ability to generate reactive oxygen species (ROS), XO/XDH plays a significant role in the pathogenesis of various organ diseases including acute lung injury. As a result of stimulation by endotoxin and inflammatory cytokines, iNOS also participates in tissue injury through high and sustained release of NO. The products of XO and iNOS, superoxide and NO, respectively, interact to form peroxynitrite, a highly toxic oxidant capable of oxidizing biological molecules and nitrating protein tyrosine residues. Recent evidence demonstrating the presence of nitrated tyrosine residues in patients with acute respiratory distress syndrome suggests that peroxynitrite may indeed be an important oxidant in human acute lung injury. Hypoxia-induced upregulation of XO/XDH mRNA has been observed in pulmonary artery endothelial cells (PAECs) (13), and lung XO/XDH activity and gene expression increase in response to hypoxia, endotoxin, and IL-1β treatment in an animal model of lung injury (12). Pharmacological inhibition of XO/XDH prevents the development of pulmonary edema after these treatments, further supporting a role for this enzyme in the pathogenesis of acute lung injury (12). New evidence was presented showing that lung iNOS is also upregulated by the combination of hypoxia, endotoxin, and IL-1β, consistent with the interaction of XO/XDH and iNOS products in this model of lung injury.

Evidence was also presented that at least part of the unexplained antioxidant activity of estrogen may occur via regulation of XO/XDH. Estradiol (10 μM for 24 h) significantly inhibited XO/XDH activity in normoxic and hypoxic PAECs. A 5- to 7-day exposure to physiological concentrations of estradiol also decreased XO and XDH activities and protein expression. The estrogen receptor (ER) type β (ER-β) was upregulated after exposure of PAECs to hypoxia for 24 h. To investigate the mechanism of estrogen-mediated effects on XO/XDH, the levels of ERs in the cells were enhanced by transfection with ER-α and ER-β, each tagged with the expression vector pEGFP-C2. Under normoxic conditions, cells expressing high levels of ER-α expression had decreased XO/XDH activity compared with that in control cells transfected with GFP alone, and XO/XDH activity in these cells did not increase in response to hypoxia. Taken together, these results suggest that the antioxidant activity of estrogen may be effected via

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3 Presented by Augustine M. Choi.

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inhibition of XO/XDH expression. It was speculated that the upregulation of ER-β in hypoxia may represent a counterregulatory mechanism in response to the upregulation of XO in hypoxia.

**ISCHEMIA-MEDIATED SIGNALING THROUGH ROS GENERATION BY FLOW-ADAPTED ENDOTHELIUM**

It was proposed that cessation of flow is detected by shear-sensitive elements in the pulmonary endothelium and translated into intracellular signals that serve to promote vasodilatation via NO release and tissue remodeling through activation of transcription factors (1, 22, 25). These responses require adequate oxygenation of pulmonary tissue, which is provided by continued ventilation during ischemia. Initiation of the signaling cascade results from rapid membrane depolarization followed by increased production of ROS (1, 3, 23). Using fluorescence microscopy with ROS-sensitive fluorophores in isolated rat and mouse lungs, Dr. Fisher presented evidence of endothelial generation of ROS in ischemia that begins within 1–2 min of flow cessation (11). ROS generation is markedly reduced by inhibitors of membrane-associated NADPH oxidase and is abolished by knockout of gp91phox, a major NADPH oxidase component (2).

Immunofluorescence studies by Dr. Fisher and his colleagues demonstrated the presence of all four major protein components of NADPH oxidase in rat and mouse pulmonary endothelia. The ischemia response system was reconstituted in vitro with bovine PAECs that had been conditioned to flow (shear stress of 1–2 dyn/cm²) for 2 days in an artificial capillary system. Cessation of flow in this vitro system led to ROS generation, activation of nuclear factor-κB and activator protein-1, increased [3H]thymidine incorporation into DNA, and cell proliferation as indicated by cell cycle analysis. These responses were not seen in control (non-flow-adapted) cells.

These studies indicated participation of endothelial NADPH oxidase and perhaps other ROS-generating pathways in the physiological response to altered pulmonary perfusion, leading to a cell signaling cascade. These events could also lead to oxidative lung injury depending on the balance between ROS generation and antioxidant defenses.

In summary, the symposium presentations reflect growing insight into the roles of intracellular (MT, XO/XDH, and HO) and plasma membrane (NADPH oxidase, XO/XDH, and unidentified TPMET) proteins in lung redox homeostasis. The focus was on the lung endothelium, but at least some of the proposed mechanisms no doubt operate in other cell types and organs as well. In addition, the redox activities present on the luminal pulmonary endothelial surface, in conjunction with the large pulmonary endothelial surface area to which the venous blood is exposed before entering the systemic arteries, suggest a possible impact of pulmon-


