Nitric Oxide and Zinc Homeostasis in Acute Lung Injury

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Among putative small molecules that affect sensitivity to acute lung injury, zinc and nitric oxide are potentially unique by virtue of their interdependence and dual capacities to be cytoprotective or injurious. Nitric oxide and zinc appear to be linked via an intracellular signaling pathway involving S-nitrosation of metallothionein—itsself a small protein known to be an important inducible gene product that may modify lung injury. In the present article, we summarize recent efforts using genetic and fluorescence optical imaging techniques to: (1) demonstrate that S-nitrosation of metallothionein affects intracellular zinc homeostasis in intact pulmonary endothelial cells; and (2) reveal a protective role for this pathway in hyperoxic and LPS-induced injury.

Keywords: hyperoxia; LPS; metallothionein; nitric oxide; pulmonary endothelium; zinc

Genome-wide screening has been applied to a number of experimental and clinical conditions, in part, to reveal candidate genes that may modify the course of the relevant pathogenesis and suggest new therapeutic targets. Metallothionein (MT), a small intracellular cysteine-rich metal-binding protein with unique antioxidant activity, has been shown to be upregulated as determined by gene profiling in murine hyperoxic lung injury (1), confirming original observations using subtraction hybridization techniques in hyperoxic rabbit lung (2). Although the functions of MT remain unclear (3), its role in metal ion homeostasis including the binding and release of zinc is apparent. We (4, 5) and others (6–8) have shown that S-nitrosation of MT results in elevations in labile zinc, suggesting a novel nitric oxide (NO)-MT-Zn signaling pathway. Zinc itself appears to have a critical role in cell death decisions and pathways, and is capable of promoting necrosis or inhibiting apoptosis depending upon the experimental conditions and environment (9–15). In the present article, we summarize our understandings of the role of zinc, MT, and NO in the setting of acute lung injury, and suggest that NO-MT-Zn signaling pathway may be especially important in the setting of such injury where abrupt changes in the levels of all these molecules is apparent.

ZINC

Zinc and Lung Biology

After iron, zinc is the most abundant trace essential metal. Unlike iron (or copper), zinc itself is redox inert. Intracellular levels of Zn are maintained by dynamic process (16) of transport,

intracellular vesicular storage, and binding to a large number of proteins (estimated at 3% of human genome [17]). As such, Zn is an integral component of numerous metalloenzymes, structural proteins, and transcription factors. In addition to its contribution in metalloregulatory enzymes and proteins, it affects the biophysical properties of cell membranes and appears to have an antioxidant function (18, 19). Although its role in lung is unclear, some insight has recently been gained in the respiratory epithelium (20) and we have begun investigating roles in pulmonary endothelium (21). Truong-Tran and colleagues (22) have suggested that in the respiratory tract, zinc may be an antioxidant, membrane and cytoskeletal stabilizer, essential component of DNA synthesis and cellular growth, participate in wound healing, and in general act as an antiinflammatory molecule. Perhaps because of its pleiotropic nature and/or simplified approaches for supplementation, Zn has become an interesting candidate for pharmacotherapy of a number of pulmonary disorders including upper airway viral infection (23) and asthma (24). Most recently, the efficacy of extracellular zinc in restoring chloride secretion across cystic fibrosis (CF) airway epithelia (25) underscores its potential as adjunct therapy in CF (26).

Zinc and Cell Death

Since our first insights into the process of apoptosis, it has been clear that zinc can act as an inhibitor of this pathway. The initial dogma was that Zn directly inhibited: (1) $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonucleases that were responsible for DNA fragmentation (27); (2) the activity of caspase-3, a critical protease in apoptosis (28); or (3) the processing of caspase-3 (12, 29). In Fas-induced apoptosis, Zn does not inhibit cytochrome c release and thus acts somewhere between cytochrome c release and caspase-3 activation, perhaps via cytochrome c interactions with caspase-9, Apaf-1, or Bcl-X_L (30). In glucocorticoid-induced apoptosis, zinc is inhibitory by blocking binding of steroids to glucocorticoid receptors (9). TPEN, a $\text{Zn}^{2+}/\text{Fe}^{2+}$ chelator with low affinity for Ca^{2+} , has been useful in revealing a role for Zn inhibition of apoptosis (31, 32), and studies with zinc-sensitive fluorophores suggest a role for labile pool of intracellular Zn in apoptosis (33). These approaches have been used by Truong-Tran and colleagues (20) to show that zinc is a survival factor in respiratory epithelium (34, 35), and we recently noted that chelation of zinc results in spontaneous apoptosis of cultured sheep pulmonary artery endothelial cells (SPAEC) (21).

In spite of the above evidence, it is also apparent that zinc can contribute to necrosis, and compelling evidence is especially apparent in the setting of excitotoxicity in the central nervous system (36). Various experimental models of excitotoxicity have shown that Zn accumulates in targeted neurons (37) via an influx of Zn from the extracellular space and is associated with oxidative stress (10, 38). In non-neuronal tissue, the Zn chelator TPEN was capable of reducing peroxynitrite toxicity, in part by inhibition of Zn-dependent nuclear enzyme, poly (ADP-ribose) synthetase (PARS), resulting in less ONOO-induced mitochondrial damage (11). It is noteworthy that like NO, zinc has a dual role in cell death pathways, and as in excitable tissue, we have shown that zinc can actually participate in necrotic cell death in lung endothelial cells (21).

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Zinc and Acute Lung Injury

Exposure to extraordinarily high concentrations of zinc (e.g., accidental inhalation of zinc chloride [39]) can produce fatal acute lung injury in humans. In addition, zinc appears to account for a significant aspect of particulate matter toxicity (40–43). Nonetheless, zinc deficiency (secondary to dietary manipulations) enhances hyperoxic lung injury (44), and exogenous zinc is effective in ameliorating hyperoxic (45) or carbon tetrachloride (46) lung injury. Accordingly, it is apparent that dependent upon its intracellular concentration and changes in the ordinarily low labile pool, zinc can be injurious or cytoprotective, and can be a component of pathogenesis or a target for therapeutic approaches.

METALLOTHIONEIN

Metallothionein (MT) is a small (6–7 kD) intracellular metal (Zn, Cu, Cd)-binding protein that is ubiquitous to eukaryotes and whose function remains unclear (3, 47). It is capable of binding essential metals such as zinc and copper as well as nonessential toxic metals such as cadmium, mercury, and silver. Its extrahepatic constitutive expression is low, but it is readily induced at a transcriptional level by a variety of stimuli including metals themselves and proinflammatory molecules (cytokines, reactive oxygen and nitrogen species). Metal-induced increases in MT gene expression reduce cellular and organismal responses to metals and result in cross resistance to oxidative stress. Accordingly, MT appears to be a stress gene clearly involved in metal detoxification and metal ion homeostasis and perhaps contributing to cellular antioxidant defense mechanisms. Studies in genetically modified mice have clearly supported a functional role for MT in protection against toxic metals (47). The latter function has also received considerable support from transgenic approaches in which overexpression of MT decreases sensitivity to oxidative and nitrosative stress (and conversely targeted deletion of MT genes in null mice enhances sensitivity) in: (1) central nervous system (48–52), (2) skin (53, 54), (3) gastrointestinal tract (55–59), (4) heart (60–64), and (5) lung (65, 66).

MT and Lung Biology

Although intrapulmonary MT levels are low (67), they are readily induced by exposure of intact animals to cadmium (68), paraquat, and tBH (69). Indeed, MT was one of the first hyperoxic sensitive genes revealed in rabbit lung by subtraction hybridization (70), and this observation has been reproduced by new techniques in gene profiling in hyperoxic lung (1, 71), as well as in acute lung injury induced by lipopolysaccharide (65), diesel exhaust particles (72), and ventilator-associated acute lung injury (73). MT also appears to be protective against ovalbumin-induced airway inflammation (66). Gene profiling in other pathologies, including experimental models of diabetes (74) and myocardial infarction (75), also has revealed MT as a candidate molecule. MT protein has been shown to be elevated in hyperoxia (2), and indeed its mRNA is used as an index of sensitivity to hyperoxic injury (76). Exposure of intact animals to aerosolized cadmium produces tolerance to subsequent exposure to high oxygen (77). Alveolar type II cells (78) and alveolar macrophages (79) isolated from rats after repeated exposure to cadmium are resistant to hydrogen peroxide. In humans, MT expression is detectable constitutively in alveolar macrophages, pleural endothelial cells, and basal cells from bronchial epithelium (80).

We noted that overexpression of MT in cultured SPAEC reduced the sensitivity to exposure to 95% oxygen (81). More recently we have noted that mice in which MTI and MTII were deleted by targeted ablation (MT^{-/-}) were more sensitive than wild-type controls (MT^{+/+}) to continuous exposure to > 99% oxygen (B. R. Pitt and coworkers, unpublished observations).

NITRIC OXIDE

Nitric Oxide, S-Nitrosation, and Zinc Sulfur Clusters

Although the reactivity of NO in biological systems is relatively low, secondary reactions involving molecular oxygen, superoxide anion, and transition metals account for much of the biological effects of NO. These reactions produce a complex mixture of reactive nitrogen oxide intermediates (RNOI) that support additional nitrosative reactions at nucleophilic centers, and accordingly most intracellular molecular targets of NO contain cysteines and/or metals (iron) at their allosteric and/or regulatory sites (82). Indeed, the ability of RNOI to S-nitrosate cysteines on over 100 proteins has led several investigators to suggest that nitrosothiols function as post-translational modifications analogous to the better accepted role of protein phosphorylation (83). The specificity of such post-translational modification is predicted to be secondary to thiol nucleophilicity (e.g., pK_a) and is affected by allosteric factors (including metal ligand interactions), hydrophobicity of the protein, subcellular localization, and complex aspects of three-dimensional protein structure (82).

In light of these latter considerations, it is not surprising that zinc sulfur clusters represent molecular targets for NO and its reactive nitrogen oxide intermediates. Kroncke, Kolb-Bachofen and colleagues have shown that NO can nitrosate various zinc dependent transcription factors and modify their contribution to gene expression (84). For example, they originally noted that NO caused the release of zinc from Sp1 and EGR-1 (85) resulting in loss of their DNA binding activity. NO also interfered with the dimerization of vitamin D3 receptor and retinoid X receptor affecting their transcriptional activity and in intact cells, NO was shown to inhibit IL-2 expression secondary to its S-nitrosation of zinc dependent transcription factors (86). These authors suggest that NO disruption of zinc fingers is an important aspect of the ability of NO to modify gene expression and outline a schema in which activation and/or inhibition may account for specificity of this novel pathway (86).

NO and Zinc Homeostasis

In addition to affecting gene expression, S-nitrosation of zinc sulfur clusters can affect intracellular metal ion homeostasis. The chemical biology underlying this process as well as other redox sensitive aspects of zinc-sulfur complexes has recently been reviewed (87). Kroncke and colleagues originally showed that NO could S-nitrosate the major intracellular zinc-binding protein, MT (84), and cause the release of zinc-detectable changes in free zinc (6). The effect appeared to be cell-specific in that NO increased detectable labile zinc in endothelial cells, whereas it reduced this signal in cultured pancreatic islet cells (88). Subsequently, Spahl and coworkers (89) noted that iNOS-derived NO increased nuclear Zn and that this increase appeared to require the translocation of MT from cytoplasm to nucleus. We (90, 91) and others (92, 93) confirmed these observations and demonstrated that: (1) S-nitrosation caused conformational changes of MT (via fluorescence resonance energy transfer techniques; Figure 1) in intact pulmonary endothelium consistent with zinc release (4, 94); (2) NO caused an increase in free zinc in pulmonary artery endothelial cells (21, 95); and (3) MT was the requisite target for NO resulting in such changes in free zinc (5). The metal status of MT was critical for resultant NO-mediated changes in that: (1) NO did not cause release of zinc in a cell in which most of the MT was in its apo form (5); and (2) Cu-MT was also nitrosated and depending upon the copper status and the amount of NO exposure, CuMT served as a copper chaperone for apo-ZnSOD (96) or a source of Fenton reactive copper (91). S-nitrosation: (1) requires the presence of molecular oxygen (92, 97), (2) is modified by redox status of the environ-

Figure 1. A schema of a fluorescence resonance energy transfer (FRET) capable reporter molecule (MT) and full spectral report of a single sheep pulmonary artery endothelial cell exposed to the NO donor, L-S-nitroso-cysteine ethyl ester (L-SNCEE). In the *upper panel*, a schema is shown describing the use of a chimera containing cDNA for human MT-IIA sandwiched between enhanced cyan fluorescence protein (ECFP) and enhanced yellow fluorescence protein (EYFP) at N- and C-termini, respectively. Exposure to NO or a metal chelator (EDTA) removes zinc from FRET-MT and causes a conformational change in which the molecule unfolds and the reporter molecules move more distant from each other. In the *lower panel*, full spectral report is shown by visualizing a single cultured pulmonary artery endothelial cell that was infected with an adenovirus containing cDNA to FRET-MT construct at control and 10 min after exposure to the membrane-permeant NO donor, L-SNCEE. Exposure to L-SNCEE resulted in a decrease in the acceptor and an unquenching of the donor peak. Full spectral reporting was accomplished via the use of a Zeiss 510Meta (Jena, Germany) confocal microscope.

ment (98), and (3) is more facile for the MT-III than other isoforms of MT (93). Collectively, it is apparent that S-nitrosation of zinc sulfur clusters is an important component of NO signaling and that metallothionein appears to be a critical link between NO and intracellular zinc homeostasis (5, 99).

NO and Apoptosis

The importance of NO-mediated cytotoxicity has been appreciated since the L-arginine-NO biosynthetic pathway was first identified in macrophages (100), and has lent credence to the concept of iNOS-derived NO as an important regulator and effector molecule during infection and inflammation affecting tumor cells, microorganisms, and host cells. In a cell-specific fashion, especially under conditions of oxidative stress (e.g., reduced levels of GSH, increased levels of peroxynitrite), NO has clearly been shown to be proapoptotic. In cells such as macrophages, thymocytes, pancreatic islet cells, and tumor cells, NO may be proapoptotic by: (1) activation of mitochondrial pathways (cytochrome c release through mitochondrial membrane potential loss), (2) induction of p53 expression (secondary to DNA damage), (3) activation of JNK/STAT and p38 kinase, or (4) activation of magnesium-dependent neutral sphingomyelinase and ceramide formation (101).

Nonetheless, since the initial observation by Mannick and coworkers (102) that iNOS expression or NO donors inhibited apoptosis in human B lymphocytes, it is now apparent that NO may be antiapoptotic (103) in a cell-specific fashion (hepatocytes, endothelial cells, neurons, eosinophils), especially when produced in low amounts and at moderate rates in a reduced cellular environment. The antiapoptotic effects of NO have been: (1) noted *in vitro* and *in vivo* (104), (2) operate in response to a variety of apoptotic stimuli (105, 106), (3) can be the result of iNOS (as well as constitutive NOS)-derived NO, and (4) involve both cGMP-dependent and -independent pathways. In this latter regard, cGMP-dependent mechanisms for NO-mediated inhibition of apoptosis are likely to be cell specific and involve PKG-mediated phosphorylation that ultimately suppresses cytochrome c release, ceramide generation, caspase activation, enhanced BCL2 production, or activation of Akt/PKB pathways (101). Relevant to this review are the increasing number of reports suggesting that S-nitrosation of critical proteins (e.g., caspases [107]) and/or NO-mediated induction of antiapoptotic stress genes (e.g., HSP70, HSP32, metallothionein [108]) are critical components of the ability of NO to inhibit apoptosis.

Important background for the current review were our original observations that direct gene transfer (retroviral or adenoviral-mediated) of iNOS or exposure to NO from chemical donors led to a time-dependent resistance of cultured pulmonary endothelial cells to LPS-induced apoptosis (109). We originally described the ability to stably infect cultured SPAEC with retroviral-mediated human iNOS (110) and not affect their baseline phenotype. Subsequently we showed that adenoviral-mediated iNOS-derived NO or chemical donors of NO reduced the sensitivity of cultured SPAEC (111) to LPS-induced apoptosis. This resistance took 48 h to occur and was associated with inhibition of LPS activation of caspase 3 (95, 105, 111). We were unable to identify the mechanism for such resistance, but it was likely to involve the effect of NO on cellular metabolism and perhaps new gene expression, as it required a minimum of 48 h to be apparent. It was not restricted to SPAEC, as cultured primary hepatocytes could be conditioned with NO to manifest a similar resistant phenotype to TNF- α (and actinomycin) induced apoptosis (112). A survey of 8,000 genes at mRNA level was undertaken under these latter conditions (108). Noteworthy were increases in several stress genes, including metallothionein. Support for a role for MT in mediating such resistance was recently noted (B. R. Pitt and colleagues, unpublished data) when exposure to an NO donor reduced the sensitivity to LPS (plus cycloheximide)-induced apoptosis in cultured pulmonary endothelial cells from wild-type, but not MT-/- mice.

NO and Acute Lung Injury

Although iNOS (113) and reactive nitrogen oxide intermediates (114) are elevated in hyperoxia and may participate in hyperoxia-induced lung injury, it is apparent that under appropriate conditions NO (including iNOS-derived NO) may limit hyperoxic lung injury (115). In this regard: (1) inhalation of NO has been shown to reduce hyperoxia-induced apoptosis in lungs of intact animals (116), (2) pharmacologic inhibition of NO synthesis exacerbates hyperoxic injury (117), and (3) iNOS null mutant mice have enhanced inflammation (accumulation of neutrophils) early in the course of hyperoxia (118). Nonetheless, the potential of NO and its secondary nitrogen oxide intermediates (e.g., peroxy-nitrite) to contribute to lung injury is also apparent (114). Indeed, Zhu and coworkers (119) reported increased levels of nitrogen monoxides in bronchoalveolar lavage of patients with permeability edema compared to those with hydrostatic edema, and such elevations were associated with decreased alveolar fluid clearance and enhanced nitration of surfactant protein A (SP A). In addition to surfactant proteins (including SP A and B [120]), other targets for NO and/or peroxynitrite include respiratory epithelial sodium channel (121) and chloride channels including cystic fibrosis transmembrane conductance regulator (CFTR) (122). As such, endogenous NO (or exogenous NO via inhalation of NO gas) has the potential to limit resolution of pulmonary edema and depress host defense of lung, thereby exacerbating acute lung injury.

CONCLUSIONS

The importance of activation of soluble guanylyl cyclase in mediating the effects of NO emerged simultaneously with the discovery of the L-arginine-dependent NO biosynthetic pathway. Nonetheless, it is becoming increasingly apparent that non-cGMP-mediated events contribute to the bioactivity of NO. In this regard, an emerging role for S-nitrosation of regulatory proteins has been revealed, and indeed analogies of the importance of such post-translational modification in signaling to the better known pathways of phosphorylation are now well documented. NO and Zinc are two small molecules that contribute to the sensitivity of the lung to acute injury, and as such represent unique components of the pathogenesis of acute lung injury as well as potential therapeutic modalities. They appear linked via a process in which NO and its reactive species (nitrosonium) can S-nitrosate MT and result in increases in labile pool of zinc. Under a variety of conditions, all three molecules (NO, MT, and Zn) can reduce the sensitivity of lung to hyperoxic and LPS-induced injury, and thus there is a need to further evaluate the physiologic significance of NO-MT-Zn signaling pathway. We and others have provided some insight into potential functions that may be modified by this novel signaling pathway, including: (1) vasomotor tone, including myogenic reflex (4) and hypoxic vasoconstriction (B. R. Pitt and colleagues, unpublished observations) in systemic and pulmonary circulation; (2) gene regulation, especially among candidate genes that are regulated by zinc-associated transcription factors (87); and (3) pathways of cell death, including those within neurons of the central nervous system (15) and pulmonary endothelium (21). The molecular subtleties and overlap with existing signaling pathways of the NO-MT-Zn pathway remain to be elucidated.

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