Contribution of Reactive Oxygen and Nitrogen Species to Particulate-Induced Lung Injury

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Recently, a second pathway for the generation of potential oxidants with the reactivity of the hydroxyl radical without the need for metal catalysis has been described. In response to various inflammatory stimuli, lung endothelial, alveolar, and airway epithelial cells, as well as activated alveolar macrophages, produce both nitric oxide (•NO) and superoxide anion radicals (O_2^-). •NO regulates pulmonary vascular and airway tone and plays an important role in lung host defense against various bacteria. However, •NO may be cytotoxic by inhibiting critical enzymes such as mitochondrial aconitase and ribonucleotide reductase, by *S*-nitrosolation of thiol groups, or by binding to their iron–sulfur centers. In addition, •NO reacts with O_2^- at a near diffusion-limited rate to form the strong oxidant peroxynitrite ($ONOO^-$), which can nitrate and oxidize key amino acids in various lung proteins such as surfactant protein A, and inhibit their functions. The presence of $ONOO^-$ in the lungs of patients with acute respiratory distress syndrome has been demonstrated by measuring levels of nitrotyrosine, the stable product of tyrosine nitration. Various studies have shown that inhalation or intratracheal instillation of various respirable mineral dusts or asbestos fibers increased levels of inducible nitric oxide synthase mRNA. In this presentation, we review the evidence for the upregulation of •NO in the lungs of animals exposed to mineral particulates and assess the contribution of reactive nitrogen species in the pathogenesis of the resultant lung injury. — *Environ Health Perspect* 106(Suppl 5):1157–1163 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-5/1157-1163zhu/abstract.html*

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Introduction

The most important function of the lung is gas exchange. The gas exchange surface is mainly composed of a single thin layer of squamous epithelial cells, the alveolar type I cells. Interspersed among these are the larger cuboidal alveolar type II cells that produce the fluid layer that lines the alveoli. A third cell type, the free-ranging phagocytic alveolar macrophage, is found in varying numbers in the extracellular lining of the alveolar surface. These cells patrol the alveolar surface and phagocytize inspired particulates such as bacteria, anthracotic pigment, and particulates in cigarette smoke. The tight junctions between epithelial cells are organized so as to provide a high-resistance barrier to fluid movement from the interstitial to the alveolar space (*1*).

The alveolar epithelium is continuously exposed to both endogenously and exogenously derived sources of reactive oxygen and nitrogen species. These reactive species are formed as intermediates in mitochondrial electron transport systems and microsomal metabolism of endogenous compounds and xenobiotics, including drugs and environmental pollutants and various cytoplasmic sources. In addition, neutrophils and other inflammatory cells generate and release reactive oxygen species via an NADPH-oxidase-dependent mechanism that is mediated by membrane receptor activation of protein kinase C and phospholipase C (*2*). It has been shown recently that overproduction of endogenous nitric oxide (•NO) by alveolar macrophages, epithelial, interstitial, and endothelial cells, as well as by inhalation of •NO, contributes to the alveolar epithelium's oxidant burden by the production of reactive oxygen–nitrogen intermediates (*3,4*).

Various studies have shown that inhalation or intratracheal instillation of respirable dusts such as silica, coal mine dust, or asbestos increased mRNA levels of inducible nitric oxide synthase (iNOS). Herein, we consider the basic biochemistry of reactive oxygen and nitrogen species, review the evidence for the upregulation of •NO in the lungs of animals exposed to asbestos fibers, and assess the contribution of reactive nitrogen species in the pathogenesis of the resulting lung injury.

Biochemistry of Reactive Oxygen and Nitrogen Species

Reactive Oxygen Species

Under normal oxygen tensions in humans, approximately 98% of oxygen undergoes a four-electron catalytic reduction to form water by mitochondrial cytochrome c oxidase. The remaining 2% of oxygen, however, may undergo sequential incomplete reduction to form reactive oxygen species such as superoxide anion radicals ($O_2^{\bullet -}$) and H_2O_2. Both $O_2^{\bullet -}$ and H_2O_2 are relatively long-lived compounds in biologic systems. H_2O_2 can directly cross cell membranes by simple diffusion, and $O_2^{\bullet -}$ crosses cell membranes via anion channels. H_2O_2 is less reactive than $O_2^{\bullet -}$ or hydroxyl radical (•OH), but it can exert toxic effects

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Abbreviations used: AG, aminoguanidine; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; IFN-γ, interferon γ; I-κBd, α isoform of inhibitory subunit of NF-κB; IL, interleukin; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor kappa B; NO^+, nitrosonium ion; •NO, nitric oxide; $•NO_2$, nitrogen dioxide; NOS, nitric oxide synthase; OH^-, hydroxide ion; •OH, hydroxyl radical; $ONOO^-$, peroxynitrite; ONOOH, peroxynitrous acid; $O_2^{\bullet -}$, superoxide anion radical; PARS, poly-ADP-ribose synthetase; RS-NO, nitrosothiol; TNF-α, tumor necrosis factor alpha; SOD, superoxide dismutase; SP-A, surfactant protein A.

more distal than either $O_2^{\cdot-}$ or $^{\cdot}OH$. However, the limited reactivity with many biological molecules and the low intracellular concentrations of $O_2^{\cdot-}$ and H_2O_2 (10 pM and 1–100 nM, respectively) have raised questions about their toxicity per se *in vivo*.

A more potent reactive metabolite of $O_2^{\cdot-}$ that is generated in a variety of biologic systems is $^{\cdot}OH$. $O_2^{\cdot-}$ directly reduces H_2O_2 (generated from the simultaneous dismutation of $O_2^{\cdot-}$) to give $^{\cdot}OH$, O_2 and hydroxide ion (OH^-; Haber-Weiss reaction). In a modified Haber-Weiss reaction (Fenton reaction), $O_2^{\cdot-}$ reduces trace metals (usually Fe^{3+}, sometimes Cu^{2+}) and generates O_2. The reduced form of the metal then reacts with H_2O_2 to generate the initial oxidized form of the metal, OH^-, and $^{\cdot}OH$. In addition to simultaneous dismutation of $O_2^{\cdot-}$, H_2O_2 may also come from superoxide dismutase (SOD)-catalyzed, which may occur as the result of the induction of SOD without corresponding upregulation of catalase. The reactivity of $^{\cdot}OH$ is so high and nonspecific that the site of target reaction is confined to within a few molecular radii of the site of its generation.

However, generation of $^{\cdot}OH$ by Fenton reaction requires the interaction of three different species ($O_2^{\cdot-}$, H_2O_2, and Fe^{3+}). In the epithelial lining fluid, the concentrations of reactive oxygen species are kept low due to the presence of the enzymes, such as SODs (CuZnSOD, found mainly in the cytoplasm as well as in peroxisomes, and MnSOD localized in the mitochondria), catalase, localized in the peroxisoms along with a number of nonenzymatic antioxidants including vitamin E and high concentrations of reduced glutathione and ascorbate (*5*). Also, an extracellular form of SOD (EC-SOD) has been localized in the lung matrix and is thought to play a major role in the scavenging of extracellular $O_2^{\cdot-}$ (*6*). Furthermore, most iron is chelated in a noncatalytic form by transferrin and ceruloplasmin in epithelial lining fluid.

Several factors may exacerbate production of reactive oxygen species in acute and chronic lung diseases. First, increased oxygen concentration is commonly required to alleviate hypoxemia. Exposure of lung cells, subcellular organelles, and tissue to hyperoxia (100% O_2) increases mitochondrial H_2O_2 production 10- to 15-fold (*7*). Second, in response to proinflammatory cytokines, activated neutrophils and macrophages migrate to the lungs and release reactive oxygen species by the membrane-bound enzyme complex NADPH oxidase (*8*). Third, under conditions of ischemia, decreased perfusion, low oxygen tension, or trauma, xanthine dehydrogenase, the innocuous form of the enzyme, is converted to xanthine oxidase, which uses xanthine and molecular oxygen to produce partially reduced oxygen species. The results of several studies suggest that xanthine oxidase may be released from the intestine or liver into the circulation and bind to pulmonary endothelium, where it can serve as a locus for the intense production of reactive oxygen species (*9*).

Reactive Nitrogen Species

Although the formation of $^{\cdot}OH$ via the Fenton reaction *in vivo* may still occur, especially in situations where the intracellular load of free iron has been increased (*10*), a second pathway for the generation of potential oxidants with the reactivity of $^{\cdot}OH$ without the need for metal catalysis has recently been described (*11*).

$^{\cdot}NO$, a signal-transducing free radical, is synthesized from the five-electron oxidation of the guanidino nitrogen of L-arginine by nitric oxide synthase (NOS). In this reaction, O_2 and NADPH act as cosubstrates, whereas tetrahydrobiopterin, flavin nucleotides FMN and FAD, and thiols serve as enzyme cofactors. N^G-hydroxy-L-arginine is formed as a short-lived intermediate and L-citrulline is the by-product (*12*). $^{\cdot}NO$ causes smooth muscle relaxation and reduces platelet and neutrophil adhesion to endothelium by activating soluble guanylate cyclase and increasing cGMP (*12*). In addition, $^{\cdot}NO$ is inactivated upon entering the blood stream because of its rapid reaction with hemoglobin (*12*). For these reasons, inhaled $^{\cdot}NO$ has been advocated as a highly selective pulmonary vasodilator. Pertinent sources of pulmonary $^{\cdot}NO$/peroxynitrite ($ONOO^-$) include activated macrophages (*3*), alveolar type II cells (*13*), endothelial cells (*14*), and airway cells (*15*). The Ca^{2+}-independent form of NOS, the enzyme responsible for $^{\cdot}NO$ formation during inflammation, has been immunolocalized to human lung tissue obtained from patients with pneumonia and sepsis (*15*). Both alveolar macrophages and type II cells can up regulate their $^{\cdot}NO$ production when exposed to diverse stimuli such as cytokines, lipopolysaccharide, and interferon γ (IFN-γ) (*13,16*), raising the possibility of increased $^{\cdot}NO$ release into the epithelial lining fluid during lung inflammation.

Because $^{\cdot}NO$ is a free radical, it will react readily with other free radicals, either detoxifying them or creating very toxic reactive species. A detailed discussion follows of the biochemical mechanisms by which interaction of $^{\cdot}NO$ with biologic targets alters their function.

The Dark Side of $^{\cdot}NO$

Although $^{\cdot}NO$ defends the host against infectious agents, its effects are nonspecific. Overproduction of $^{\cdot}NO$ may be cytotoxic not only for microbes but also for the cells and tissues that produce it (*17*). At high concentrations, $^{\cdot}NO$ inactivates critical enzymes by interacting with their iron-sulfur centers (*18*), causes DNA strand breaks that result in the activation of the nuclear enzyme poly-ADP-ribosyl transferase (*18*), and inhibits both DNA and protein synthesis (*19,20*). However, most of the toxic effects of $^{\cdot}NO$ have been attributed to its reaction with $O_2^{\cdot-}$, with a rate constant of about 7×10^9 $M^{-1}\cdot sec^{-1}$ to form $ONOO^-$. The protonated form of $ONOO^-$, peroxynitrous acid (ONOOH), forms nitrogen dioxide ($^{\cdot}NO_2$) and an intermediate with reactivity equivalent to the $^{\cdot}OH$ derived from the *trans*-isomerization of ONOOH, as shown in Equation 1 (*11*):

$$O_2^{\cdot-} + {}^{\cdot}NO \rightarrow ONOO^- + H^+ \rightarrow ONOO \rightarrow [{}^{\cdot}OH \cdots {}^{\cdot}NO_2] \quad [1]$$

$ONOO^-$ initiates iron-independent lipid peroxidation and oxidizes thiols at rates at least 1000-fold greater than that of H_2O_2 at pH 7, damages the mitochondria electron transport chain, and causes lipid peroxidation of human low density lipoproteins. $ONOO^-$-mediated thiol oxidation occurs at physiologic pH and in some cases may be irreversible, i.e., oxidized sulfhydryl groups cannot be reduced by physiologic reductants. In addition, $ONOO^-$ nitrates phenolics, including tyrosine and tryptophan residues in several proteins (*21*). Using luminol-dependent chemiluminescence, $ONOO^-$ production has been demonstrated by human neutrophils (*22*), rat alveolar macrophages (*3*), and bovine aortic endothelial cells (*14*).

It can be argued that the alveolar epithelial lining fluid contains a number of antioxidant substances such as SODs, catalase, reduced glutathione, urate, etc. that will limit the steady-state concentrations of reactive oxygen and nitrogen species *in vivo*. However, footprints of $ONOO^-$ have been identified in a number of organs, including lungs of infants who died with respiratory failure (*23*). Furthermore,

because of its high reactivity, $ONOO^-$ will attack biologic targets even in the presence of antioxidant substances (*24*). Recent reports (*25,26*) indicate that physiologic concentrations of carbon dioxide and bicarbonate enhance the reactivity of $ONOO^-$ via the formation of the nitrosoperoxycarbonate anion ($O=N-OOCO_2^-$) and increase the yield of nitration. Equally important, bicarbonate reverses the inhibition of $ONOO^-$-induced nitration by ascorbate and urate (*26*). Also, the reaction of nitrite, the stable by-product of $^{\bullet}NO$ and $ONOO^-$, with hypochlorous acid forms reactive intermediate species that are also capable of nitrating tyrosine with maximum yield at pH 7.4 (*27*). The detection of nitrotyrosine in the lungs of patients with acute respiratory distress syndrome (*23*), and lungs of rats exposed to endotoxin (*28*) or hyperoxia (*23*) indicates that nitration reactions occur *in vivo*. Our recent findings and those of others indicate that *in vivo* injury to the alveolar epithelium and pulmonary surfactant system, previously attributed to reactive oxygen species, may be caused instead by reactive oxygen–nitrogen intermediates such as $ONOO^-$ (*29,30*).

Physiologic Consequences of Protein Nitration

Several reports indicate that protein nitration may lead to loss of function. Nitration of tyrosine residues of human IgG, but not rabbit IgG, abrogated their C1q-binding activity (*31*). This was consistent with the presence of a tyrosine residue at the C1q receptor site of human but not rabbit IgG. The inactivation of *Escherichia coli* dUTPase and the occurrence of a tyrosine residue in a strictly conserved sequence motif suggest the critical importance of this residue for the function of the enzyme (*32*). Nitration of tyrosine residues of α_1-proteinase inhibitor resulted in selective loss of elastase inhibitory activity but not chymotrypsin or trypsin inhibitory activity (*33*). Tyrosine nitration also inhibits protein phosphorylation by tyrosine kinases, which may interfere with intracellular signal transduction (*34*). Exposure of surfactant protein A (SP-A) to tetranitromethane or $ONOO^-$ led to nitration of a single tyrosine residue in its carbohydrate recognition domain and diminished the ability of SP-A to aggregate lipids and bind to mannose (*30,35*).

The Good Side of $^{\bullet}NO$

$^{\bullet}NO$ can ameliorate tissue injury by: *a*) activating guanylate cyclase with subsequent induction of cGMP-dependent effects such as reducing platelet and neutrophil adhesion to endothelium (*36*); *b*) decreasing $O_2^{\cdot -}$ steady-state levels, especially under conditions favoring $O_2^{\cdot -}$-dependent $^{\bullet}OH$ formation (*37*); *c*) binding to the free coordination sites of heme-bound iron, thus indirectly acting as an iron chelator (*38*); *d*) inducing glutathione (*39*); *e*) reacting with tyrosyl radicals with a rate constant of $1 \pm 0.3 \times 10^9$ $M^{-1} \cdot sec^{-1}$, thus limiting the extent of nitrotyrosine formation (*40*); and *f*) inhibiting oxidant-induced membrane and lipoprotein oxidation by annihilation of lipid radical species, thus terminating radical chain propagation reactions (*41*). This last property may act as a two-edge sword, as species resulting from the reaction of $^{\bullet}NO$ with lipid peroxides may be toxic themselves.

Reaction of $^{\bullet}NO/ONOO^-$ with Thiols

The direct reaction of $^{\bullet}NO$ with thiol groups is kinetically unfavorable and requires the nearby presence of a strong electron acceptor such as Fe^{3+}. However, $^{\bullet}NO$-derived species such as nitrosonium ion (NO^+) and $ONOO^-$ may readily react with thiols to form nitrosothiols (RS–NO). The detection of RS–NO in human bronchoalveolar lavage *in vivo* and their significant increase in the lungs of patients with pneumonia or during inhalation of 80 ppm $^{\bullet}NO$ (*42*) provide evidence that such chemical modification of protein thiols (i.e., addition of NO^+) do occur and may be a fundamental signal transduction pathway. Nitrosylation of the *N*-methyl-D-aspartate receptor in the brain leads to decreased calcium transport and neuroprotection (*43*). On the other hand, nitrosylation of glyceraldehyde-3-phosphate dehydrogenase stimulated the apparent auto-ADP ribosylation and inhibited enzymatic activity (*18*).

$^{\bullet}NO$ Modulation of Gene Expression

$^{\bullet}NO$ both inhibits and activates gene expression. $^{\bullet}NO$ decreases cytokine-induced endothelial cell activation by inhibiting nuclear factor kappa B (NF-κB) expression and subsequently either vascular cell adhesion molecule transcription (*44*) or NOS-II expression itself (*45*). $^{\bullet}NO$ affects not only NF-κB function (in part by altering I-κBα [α isoform of inhibitory subunit NF-κB] expression) but other redox sensitive transcription factors including AP-1 components (*46*). $^{\bullet}NO$ may inhibit gene expression by various mechanisms: first, it can result in DNA deamination and strand breaks with the activation of the nuclear enzyme poly-ADP-ribose synthetase (PARS) (*18*). This activated PARS catalyzes the attachment of ADP-ribose units to nuclear proteins with resultant depletion of energy stores. Second, stimulated macrophages produce enough $^{\bullet}NO$ to inhibit ribonucleotide reductase, the enzyme that converts ribonucleotides to the deoxyribonucleotides necessary for DNA synthesis (*47*). Third, key thiols of numerous transcription factors critical for DNA binding (e.g., zinc-finger proteins) can be modified by $^{\bullet}NO$ and ONOO– (*48*). Fourth, $^{\bullet}NO$ may inhibit mitochondrial respiration by interacting with iron-sulfur centers and inactivation of critical enzymes (such as mitochondrial aconitase, NADH:ubiquinone oxidoreductase), leading to energy depletion and decreased protein synthesis (*49*). However, in other studies, $^{\bullet}NO$ directly enhanced gene activity by eliciting nuclear translocation of NF-κB (*50*) and of the AP-1 subunits *c-fos*, and *jun*B (*46*).

Involvement of Reactive Oxygen and Nitrogen Species in Asbestos-Induced Lung Injury

Mineral dust particles such as silica, coal dust, and asbestos, when inhaled in sufficient quantities, can induce pulmonary injury (*51*). Three varieties of asbestos have been used commercially in North America and Europe for most of this century: chrysotile (a serpentine asbestos that has the chemical formula $3MgO \cdot 2SiO_2 \cdot 2H_2O$), and the amphiboles crocidolite ($Na_2O \cdot Fe_2O_3 \cdot 3FeO \cdot 8SiO_2 \cdot H_2O$) and amosite ($7FeO \cdot 7MgO \cdot 8SiO_2 \cdot H_2O$). Although both silica and asbestos dusts have long been known to induce interstitial pulmonary fibrosis, the effects of asbestos are more complex because of its capacity to induce both pleural and parenchymal fibrosis (parietal pleural plaques, visceral pleural fibrosis, and asbestosis) and/or neoplasia (malignant pleural mesothelioma and bronchogenic carcinoma) (*51*). Although it is generally acknowledged that all commercial types of asbestos can induce lung cancer, there has been a long-standing debate regarding the relative potential of different mineralogic types of asbestos (chrysotile vs amphiboles) to cause pleural injury (*52,53*).

The ability of mineral dusts to induce pulmonary injury is dependent upon a number of variables, including the physicochemical and surface properties of the mineral particles, their solubility and

biodurability, the duration and severity of dust exposure, the efficacy of pulmonary particle clearance mechanisms, and variable host susceptibility factors. Using a rat tracheal organ culture system, it has been demonstrated that exposure to cigarette smoke enhanced the uptake of amosite asbestos fibers by tracheal epithelial cells (*54,55*). Cigarette smoke-enhanced uptake of amosite fibers was abrogated by the iron chelator deferoxamine and by scavengers of reactive oxygen species such as SOD and catalase. These findings suggest that reactive oxygen species (which are known to be present in tobacco smoke) may be implicated in the uptake of asbestos fibers by airway epithelium.

There is considerable evidence, based on both clinical and experimental studies, that alveolar macrophages play a key role in mediating the tissue responses to inhaled mineral particles, as these phagocytic cells are recruited to the sites of particle deposition (*56,57*). Although this has been shown with respect to asbsestos fibers as well as nonfibrous particulates such as silica and coal dust (*58–60*), it is conceivable that fiber geometry may play a role in mediating the biologic effects of asbestos in this regard. Inhalation of crocidolite or chrysotile asbestos fibers stimulated the recruitment of macrophages to the rat pleural space (*61*). A number of studies have demonstrated that both *in vitro* and *in vivo* asbestos exposure upregulate alveolar macrophage functional activity (*62,63*), as evidenced by increased synthesis of arachidonic acid metabolites (leukotrienes B_4 and C_4, 5-hydroxyeicosatetraenoic acid, and prostaglandins E_2 and $F_{2\alpha}$). Asbestos fibers activate both rat and human alveolar macrophages to secrete the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) (*64,65*). Inhalation of asbestos fibers also upregulates TNF-α secretion by rat pleural macrophages (*61*).

Particulate exposure induces the generation of reactive oxygen species in alveolar macrophages and other cells in the respiratory tract. Both chrysotile and crocidolite stimulate the *in vitro* production of $O_2^{\cdot -}$ by human alveolar macrophages (*58*). A similar observation was noted in crocidolite-exposed rat alveolar macrophages (*66*) and chrysotile-exposed guinea pig alveolar macrophages (*67*), an effect involving the opening of calcium ion channels within the macrophage (*67*).

It has been postulated that asbestos fibers also may induce the formation of $^{\cdot}OH$ and H_2O_2 via the Fenton reaction (*68*). The potential *in vivo* significance of these observations was underscored by a study that demonstrated that the administration of polyethylene glycol-conjugated catalase via an osmotic pump inhibited the development of crocidolite-induced pulmonary inflammation and interstitial fibrosis in a rat inhalation model of asbestosis (*69*). The ability to induce the formation of reactive oxygen species is not unique to asbestos fibers, as this property has also been shown for silica (*70*) and coal mine dust (*59*).

Evidence for the involvement of reactive oxygen species in the pathogenesis of asbestos-induced injury is the upregulation of antioxidant enzyme gene expression and induction of antioxidant enzyme activity. Total SOD activity increased in hamster tracheal epithelial cells exposed for several days to either crocidolite or crysotile asbestos (*71*). Lungs of rats exposed to crocidolite asbestos showed increases in the activity of all measured antioxidant enzymes, including catalase, glutathione peroxidase, and total SOD, with minor variations in the extent and time course of these increases (*72*). These elevations in enzyme activities correlate with induction of gene expression for these enzymes, with MnSOD exhibiting the most dramatic increases in steady-state mRNA levels. Elevations in MnSOD gene expression were even observed 10 to 14 days after cessation of exposures (*72*). In addition, elevations in MnSOD mRNA correlated with increases in immunoreactive protein after 20 days of exposure. Message levels of CuZnSOD, glutathione peroxidase, and catalase were also upregulated (*72*). Immunocytochemical localization studies in crocidolite-exposed rats demonstrate that MnSOD protein is expressed in the mitochondria of alveolar type II cells. Other lung cell types such as fibroblasts, alveolar macrophages, or endothelial cells contain little or no detectable immunoreactive protein (*73*), supporting the hypothesis that type II cells are more resistant to certain types of oxidative stress because of induction of MnSOD or other antioxidant enzymes.

Although Fenton reactions may be implicated in asbestos-induced injury, they are not sufficient to account for the similar fibrogenic effects of crocidolite and chrysotile, as crocidolite has approximately 18 times the elemental iron concentration of chrysotile (*60*). Conceivably, other reactive species also may play a role. Attention has focused recently on the putative role of reactive nitrogen species in this regard. One study demonstrated that both crocidolite and chrysotile asbestos fibers upregulated the production of $^{\cdot}NO$ by cultured rat alveolar macrophages in the presence of IFN-γ (*74*). Furthermore, the interaction of asbestos fibers and IFN-γ was synergistic in stimulating $^{\cdot}NO$ production (*74*). In contrast, carbonyl iron, a nonfibrogenic particulate, did not induce $^{\cdot}NO$ formation (*74*). In another study, intratracheal instillation of the pneumotoxic dusts—silica and coal mine dust—into rats caused more inflammation and $^{\cdot}NO$ formation (when normalized for equal numbers of particles) than similar administration of carbonyl iron or titanium dioxide particles (*75*). Collectively, these observations indicate that it is both the physical and chemical makeup of the mineral particle that may define its capacity to induce $^{\cdot}NO$ production. Also, because cytokine secretion is an integral component of the *in vivo* inflammatory response to mineral dusts (*64,76*), upregulation of iNOS within alveolar macrophages may occur both directly (i.e., from contact with the offending dust) and indirectly (i.e., via IFN-γ and TNF-α induction). This is of importance not only in the context of bronchoalveolar inflammation but also in the setting of pleural space inflammation, as a recent study in the rat has shown that inhalation of either crocidolite or chrysotile asbestos stimulates the secretion of TNF-α as well as the formation of $^{\cdot}NO$ by pleural macrophages (*77*).

Recent studies using human lung epithelial (A549) cells indicate that crocidolite treatment results in the formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in DNA, synthesis of mRNA for iNOS, and increased intracellular nitrite production (*10*). Aminoguanidine (AG), an NOS inhibitor, reduces intracellular nitrite and prevents 8-OHdG formation in DNA of crocidolite-treated cells. Addition of the $^{\cdot}NO$ donor diethyltriamine NONOate with AG and crocidolite results in recovery of 8-OHdG (*10*). Thus, the participation of $^{\cdot}NO$ in the oxidation of DNA may be important for the carcinogenicity of asbestos. This fact also may be pertinent in the context of pleural injury, as a recent study demonstrated that the addition of either chrysotile or crocidolite asbestos fibers to cultured rat pleural mesothelial cells, when combined with IL-1β, significantly stimulated the

formation of •NO (*77*). Notably, this effect was greater after chrysotile than after crocidolite exposure and was not observed after carbonyl iron challenge.

In addition to the diverse modulating effects of •NO per se, the results of our recent study indicate that asbestos inhalation induces iNOS in lung inflammatory and epithelial cells, which results in $ONOO^-$ formation *in vivo* (*78*). Rats were exposed intermittently over 2 weeks to either filtered room air (sham-exposed) or to chrysotile or crocidolite asbestos fibers, and were sacrificed at 1 or 6 weeks after exposure. At 1 week, significantly greater numbers of alveolar and pleural macrophages from asbestos-exposed than from sham-exposed rats demonstrated iNOS protein immunoreactivity. Alveolar macrophages from asbestos-exposed rats also generated significantly greater nitrite than macrophages from sham-exposed rats. Significant amounts of nitrotyrosine, a marker of $ONOO^-$ formation, was seen in lungs from chrysotile- and crocidolite-exposed rats at 1 and 6 weeks. Staining was most evident at alveolar duct bifurcations and within bronchiolar epithelium, alveolar macrophages, and the visceral and parietal pleural mesothelium. Lungs from sham-exposed rats demonstrated minimal immunoreactivity for nitrotyrosine. Significantly greater quantities of nitrotyrosine were also detected by quantitative enzyme-linked immunosorbent assay in lung tissues from asbestos-exposed than from sham-exposed rats (chrysotile: 84 ± 12 pmol/mg protein; crocidolite: 59 ± 5; sham (filtered air): 31 ± 3.6; numbers are means $\pm$ 1 SEM; $n = 3$). In addition, Saleh et al. (*79*) reported the existence of significant amounts of nitrotyrosine and iNOS in macrophages, neutrophils, and alveolar epithelial cells of patients with idiopathic pulmonary fibrosis and proposed that $ONOO^-$ may be responsible for the generation of fibrosis. Apoptotic mechanisms also may be implicated in asbestos-related injury, as $ONOO^-$ can mediate apoptosis (*80*), and asbestos fibers induce apoptosis in pleural mesothelial cells (*81*).

In summary, existing evidence suggests that when stimulated with inflammatory agents, alveolar macrophages and lung epithelial cells of humans and rats produce $O_2^{\bullet -}$ and •NO *in vivo* and *in vitro*. These two agents react very rapidly to form $ONOO^-$, a potent oxidizing and nirating species. Significant levels of nitrotrotyrosine have been identified in the pleura and lung parenchyma of rats that inhaled either chrysotile or crocidolite asbestos fibers. Nitrotyrosine formation alters important functions of a number of proteins, including inhibition of protein phosphorylation by tyrosine kinases, which may interfere with intracellular signal transduction. The induction of reactive nitrogen species by both chrysotile and crocidolite fibers *in vivo* may provide an alternative mechanism of asbestos-induced injury to that believed to be induced by Fenton reactions.

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