Abstract
Asbestos exposure is strongly associated with the development of malignant mesothelioma, yet the mechanistic basis of this observation has not been resolved. Carcinogenic transformation or tumor progression mediated by asbestos may be related to the generation of free radical species and perturbation of cell signaling and transcription factors. We report here that exposure of human mesothelioma or lung carcinoma cells to nitric oxide (NO) in the presence of crocidolite asbestos resulted in a marked decrease in intracellular nitrosation and diminished NO-induced post-translational modifications of tumor-associated proteins (hypoxia-inducible factor-1α and p53). Crocidolite rapidly scavenged NO with concomitant conversion to nitrite (NO2−). Crocidolite also catalyzed the nitration of cellular proteins in a phenotypic cellular changes through two processes: (a) by directly reducing bioactive NO levels and preventing its subsequent interaction with target molecules and (b) by increasing oxidative damage and protein modifications through NO2− production and 3-nitrotyrosine formation.

Introduction
Asbestos exposure is the primary cause of malignant mesothelioma. Although the peak of asbestos use in the United States occurred in 1973, it is still widely used in developing countries and it is estimated that 100,000 deaths a year occur worldwide from asbestos-related disease (1). Because the clinical appearance of malignant mesothelioma can be >40 years (2), asbestos exposure, from a public health standpoint, is still of major concern.

Asbestos is a generic term applied to a group of carcinogenic fibrous minerals. Of the four forms of asbestos, crocidolite has the greatest capacity to induce mesothelioma (3). Asbestos can regulate a variety of cell signaling pathways by stimulating gene expression and posttranslational protein modifications (4). Both in vitro (5) and in vivo (6) studies have shown that asbestos can up-regulate nitric oxide (NO) synthase (NOS), with the subsequent generation of NO and formation of protein nitrotyrosine adducts. NO regulates key tumor-related proteins in a concentration-dependent manner (7), which can either positively or negatively regulate growth.

Asbestos fibers are composed of crystalline-hydrated silicates containing various metal countercations, with iron being the most prevalent (>27%, crocidolite [Na2(Fe3+)2(Fe2+)3-Si8O22(OH)2]; ref. 8). This iron is thought to account for much of the biochemical effects of asbestos by means of direct free radical generation via Fenton-type chemistry (9). Because NO is known to participate in various metal-mediated free radical reactions, we set out to examine the chemical and biochemical implications of the reactions between crocidolite and NO on tumor-related signal transduction mechanisms.

Materials and Methods

NO measurements. NO gas (Matheson, Montgomeryville, PA) was scrubbed of higher nitrogen oxides by passage through a 1 mol/L deaerated KOH solution. The purified NO was collected by saturating a deaerated phosphate buffer solution [0.1 mol/L potassium phosphate (pH 7.4)], contained in a glass sampling flask with septum. The saturated NO solution was routinely assayed to ensure no significant contamination with higher nitrogen oxides and to determine the NO concentration, which was 1.6 to 1.9 mmol/L. NO concentrations were determined spectrophotometrically after reaction with 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (660 nm, ε = 12,000 mol/L/cm). Electrochemical analysis was accomplished using an NO-selective electrode (Apollo 4000, World Precision Instruments, Sarasota, FL). NO gas analysis was accomplished using a Seivers (Boulder, CO) NO gas analyzer. (Z)-1-[(3-amino(propionyl)]-N-[4-(3-amino(propionylammonio) butyl]-amino)diazin-1-ium-1,2-diolate (Sper/NO) was a generous gift from Drs. J.S. Seiver and J.A. Hrabie (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). Aliquots of medium (100 μL) from Sper/NO–treated cells were injected into the reaction chamber containing 0.5 mmol/L NaOH to stop Sper/NO decomposition and it was continuously purged with He gas to eliminate NO autooxidation. Steady-state molar NO concentrations were calculated from the absolute amounts of NO detected. The stock concentration of Sper/NO was determined immediately before use by measuring the absorbance at 250 nm (ε = 8,000 mol/L/cm).

Western blot analysis. Protein samples [1.4 μg of bovine serum albumin (BSA) and 50 μg total protein for hypoxia inducible factor 1α (HIF-1α) and p53 phospho-serine 15 (p53 P-Ser-15)] were subjected to PAGE on 10% Tris-glycine acrylamide gels (Novex-Invitrogen, Carlsbad, CA). Following transfer to polyvinylidene difluoride Immobilon-P membranes (Millipore, Bedford, MA), samples were probed with rabbit polyclonal 3-nitrotyrosine antibodies (0.5 μg/mL, Upstate Biotechnologies, Inc., Waltham, MA) or HIF-1α and p53 P-Ser-15 antibodies (Cell Signaling, Danvers, MA).

Nitrite detection. Nitrite was detected by the Griess reaction as previously described.

Cell culture. JMN, Meso-211H mesothelioma, A549 lung cancer, and human breast mammary gland epithelial adenocarcinoma cells (MCF-7; American Type Culture Collection, Manassas, VA) were used.

Fiber preparation. Crocidolite asbestos (National Institute of Environmental Health Sciences) was a generous gift from Dr. A.R. Brody (Department of Pathology, Tulane University Health Sciences Center, New Orleans, LA). Crocidolite was autoclaved and diluted into sterile PBS to a

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final concentration of 1 mg/mL. It was then sheared by passage through 18-gauge needles ($\times 20$) followed by sonication for 1 hour to give approximate dimensions of length of 3 to 5 $\pm$ 1.0 mm, with a diameter of 0.22 mm. Iron content of crocidolite asbestos was determined by reduction with ascorbate followed by reaction with 1,10-phenanthroline monohydrate.

Absorbance was measured at 510 nm ($\varepsilon = 11,000$/cm/mol/L). Glass Wool fibers (Corning Incorporated, Acton, MA) microscopically of similar size to crocidolite fibers were used as negative controls (1–100 µg/mL). They were composed of borosilicate glass and contained no reduct-oxide based trace metals.

**Cell viability.** Cell survival/proliferation was assessed using the nontoxic dye–based (alamarBlue) assay. When JMN mesothelioma cells were treated for 16 hours with crocidolite (1, 10, and 100 µg/cm$^2$) $\pm$ 100 µmol/L, NO$\_2$/H$_2$O$_2$, we observed decreases in cell viability of 5.8 $\pm$ 2%, 6.5 $\pm$ 2.3%, and 17.17 $\pm$ 5%, respectively compared with asbestos-free controls.

**Immunofluorescence.** Briefly, cells were rinsed and fixed with 100% methanol. Following blocking with 5% BSA, cells were incubated with 3-nitrotyrosine antibody (1:100), washed, and incubated with Alexa Fluor 488 secondary antibody.

**Nitrification.** Nitrification of human serum albumin (80 µg/mL, Sigma, St. Louis, MO) was examined following exposure to NO$_2$, H$_2$O$_2$, ATP, ascorbate (Sigma), and crocidolite or glass wool fibers (negative control). The reactions were carried out in 1-mL PBS in 5-mL glass test tubes incubated at 37°C for 2 hours. Internal 3-nitrotyrosine–positive controls consisted of BSA reacted with synthetic peroxynitrite (5 µmol/L, data not shown). Synthetic peroxynitrite was prepared by simultaneously mixing solutions of 0.5 mol/L NaNO$_2$ in 0.5 mol/L HCl and 0.5 mol/L H$_2$O$_2$ followed by rapid quenching in 1 mol/L NaOH as previously described (10).

**Fluorescence measurements.** Fluorescence measurements were obtained on a Perkin-Elmer Life Sciences (Wellesley, MA) LS50B fluorometer with excitation at 495 nm and emission at 515 nm with 5.0-nm slit widths. The reaction solution (2 mL) was stirred and maintained at 37°C with a water-jacketed cuvette holder.

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**Results and Discussion**

Asbestos is known to adsorb NO by varying degrees depending on the type of fiber (11). In the absence of crocidolite, the concentration of NO in aqueous solution decreased via the second-order reaction with oxygen ($k \approx 1.1 \times 10^{-7}$/mol/L$^2$/s; Fig. 1A, inset; ref. 12). The rate of NO disappearance was dramatically increased upon the addition of crocidolite and displayed first-order kinetics (Fig. 1A). The iron content of the crocidolite was 27 $\pm$ 4%. The rate of NO disappearance in the presence of crocidolite was further accelerated by the addition of hydrogen peroxide (H$_2$O$_2$) and ascorbate (added alone or in combination). When the metal chelator diethylenetriaminopentaacetic acid (DTPA) was added, the rate of catalytic consumption of NO by crocidolite was significantly reduced (data not shown). Taken together, these data show that crocidolite-mediated NO consumption occurs through Fenton-type chemistry via the formation of a hypervalent metal-oxo species, such as Fe(IV)O.

Real-time kinetic product analysis revealed that crocidolite catalyzed an instantaneous and stoichiometric accumulation of nitrite (NO$_2$), which paralleled the rapid disappearance of NO (Fig. 1B). This is consistent with other reports of NO metabolism by peroxidases (13). Crocidolite accelerates the rate of NO disappearance and NO$_2$ formation through several possible reactions (Eqs. A–C).

$$\text{Fe(II) + H}_2\text{O}_2 \rightarrow \text{Fe(IV)O or Fe(III) + H}_2\text{O}_2 \rightarrow \text{Fe(V)O} \quad (A)$$

$$\text{Fe(IV)O + NO} \rightarrow \text{Fe(II) + NO}_2 \quad \text{or}$$

$$\text{Fe(IV)O + NO} \rightarrow \text{Fe(III) + NO}_2 \quad (B)$$

$$2\text{Fe(III) + ascorbate} \rightarrow 2\text{Fe(II) + dehydroascorbate} \quad (C)$$

There are numerous examples of in vivo 3-nitrotyrosine staining around asbestos lesions (14, 15). Some of these reports attribute the
presence of 3-nitrotyrosine as proof of peroxynitrite (ONOO\(^-\)) formation, whereas others suggest that it represents the action of myeloperoxidases. We were the first to report that 3-nitrotyrosine could be formed nonenzymatically by \(\text{NO}_2\)/\(\text{H}^+\), \(\text{H}_2\text{O}_2\), and free metals (10). To test whether asbestos, by virtue of its high iron content, similarly catalyzes nitration, human serum albumin was incubated in the presence of \(\text{NO}_2\)/\(\text{H}^+\), \(\text{H}_2\text{O}_2\), and crocidolite. These conditions resulted in substantial 3-nitrotyrosine adduct formation (Fig. 2A). When glass wool fibers of similar size and concentration (1–100 \(\mu\text{g/mL}\)) were substituted for asbestos, no 3-nitrotyrosine was detected (Fig. 2A).

Nitrogen dioxide is both a powerful oxidant and an excellent nitrating species. Because this reaction is dependent on iron availability, we examined the influence of a biological chelator on this reaction. ATP and ADP, which are ubiquitous in cells, can chelate metals and have been shown to mobilize iron from asbestos (16). This leaching of iron has been purported to account for many of the biochemical and pathologic effects of asbestos. Addition of ATP to this reaction amplified the amount of 3-nitrotyrosine (Fig. 2A), which we attributed to an increase in the amount of labile catalytic iron. In the absence of \(\text{H}_2\text{O}_2\), nitrite and the formation of reduced metal species by ascorbate similarly resulted in protein 3-nitrotyrosine formation. The ability of crocidolite to catalyze the formation of 3-nitrotyrosine was further shown using two mesothelioma (JMN, Mesto-211H) and lung carcinoma cell lines (A549; Fig. 2B–D; JMN cells shown). When the metal chelator DTPA was present during the treatment period, substantially less 3-nitrotyrosine was observed (Fig. 2B), reconfirming the importance of redox-active iron in this reaction. These data show the susceptibility of cells to nitration by iron-rich crocidolite in a dose-dependent manner when \(\text{NO}_2\)/\(\text{H}_2\text{O}_2\) are present and that this protein adduct can be readily formed in the absence of peroxynitrite or myeloperoxidase.

Numerous tumor-associated proteins are posttranslationally regulated by discrete threshold concentrations of NO (7). Because crocidolite catalytically consumes NO, the proteins HIF-1\(\alpha\) and

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**Figure 2.** Western blot analysis of protein 3-nitrotyrosine formation by crocidolite. **A,** albumin (80 \(\mu\text{g/mL}\)) in PBS (pH 7.0) was incubated for 2 hours at 37°C in the presence of NaNO\(_2\) (100 \(\mu\text{mol/L}\)), \(\text{H}_2\text{O}_2\) (100 \(\mu\text{mol/L}\)), ascorbate (10 \(\mu\text{mol/L}\)), ATP (10 \(\mu\text{mol/L}\)), and crocidolite (0.97–10 \(\mu\text{g/mL}\)). JMN cells were plated on two-well culture slides and treated for 8 hours. HSA, human serum albumin. 3-NT, 3-nitrotyrosine. **B,** quantification of 3-nitrotyrosine formation in JMB cells treated with \(\pm\) crocidolite (1 or 10 \(\mu\text{g/cm}^2\)), NO\(_2\) (100 \(\mu\text{mol/L}\)), \(\text{H}_2\text{O}_2\) (100 \(\mu\text{mol/L}\)), and DTPA (50 \(\mu\text{mol/L}\); as indicated). **C,** untreated control. **D,** NaNO\(_2\) (100 \(\mu\text{mol/L}\)) + \(\text{H}_2\text{O}_2\) (100 \(\mu\text{mol/L}\)) + crocidolite asbestos (1 \(\mu\text{g/cm}^2\)). Cells were fixed and incubated with 3-nitrotyrosine primary and Alexa Fluor 488 secondary antibody.
p53, which are dose-dependently regulated by NO, were examined in JMN, A549, and MCF-7 cells. Figure 3A shows the correlation between the dose of NO and the degree of posttranslational regulation of HIF-1α and p53. Lung A549 cells were treated with NO for 2 hours and end point measurements were taken of steady-state NO concentrations in the medium. There was a discrete dose-dependent association between the concentration of NO and the degree of posttranslational regulation of p53 and HIF-1α. HIF-1α accumulated in response to as little as 25 μmol/L Sper/NO (≥25 nmol/L [NO]ss), whereas p53 P-Ser-15 required 50 to 100 μmol/L Sper/NO (≥200 nmol/L [NO]ss).

Because asbestos can scavenge NO and alter its steady-state concentration, NO-regulated proteins should respond differentially in the presence of crocidolite. The presence of crocidolite during NO exposure markedly diminished the extent of protein responses. JMN cells were treated with the NO donor Sper/NO (10–100 μmol/L) for 2 hours in the presence and absence of crocidolite. Figure 3B and C shows that crocidolite dramatically altered the NO-mediated posttranslational response of both HIF-1α and p53 P-Ser-15. The increases in HIF-1α levels following NO treatments (Sper/NO 10–100 μmol/L) were markedly suppressed if crocidolite fibers were present during NO exposure. Because in JMN cells, p53 is already constitutively phosphorylated at the serine-15 residue, we examined another cell line (MCF-7) where p53 is not basally phosphorylated. When these cells were similarly treated with 50 μmol/L Sper/NO, the response of both HIF-1α and p53 to NO was abated in the presence of crocidolite (Fig. 3C). These results confirm the notion that crocidolite asbestos can change phenotypic expression by directly altering the local concentration of an important signaling molecule (i.e., NO).

Having established that crocidolite catalytically consumes NO and alters signal transduction responses to NO, it was important to ascertain if this outcome was due to a direct reduction in the reactions of NO with intercellular targets. 4,5-Diaminofluorescein-diacetate (DAF-2), a nitrogen oxide–reactive fluorophore, was used to quantify in real-time the effects of crocidolite on the development of NO-mediated intracellular fluorescence. When JMN cells were preloaded with DAF-2 and exposed to NO (5 μmol/L Sper/NO) for 90 minutes, a steady increase in DAF fluorescence was observed over time (Fig. 4). In the presence of crocidolite, the quantum yield was considerably reduced, achieving only 30% of what was obtained in its absence. These results further confirm that the decreases in posttranslational responses to NO when crocidolite was present were a direct result of reduced intracellular NO exposure.

Asbestos has been shown by numerous investigators to produce free radicals under various conditions. We attribute these radicals and/or changes in the oxidation state of the iron to the reactivity of asbestos fibers toward NO. These data show that asbestos is capable of altering the local redox environment through metal-catalyzed reactions. The consequences of this are nitrite formation, nonenzymatic formation of protein nitrotyrosine adducts, and the perturbation of cell signaling events. These data, in combination with recent findings showing nitrite to be a distinct and important signaling molecule (17), suggest that asbestos could dramatically alter cell signaling events through a variety of means. Most significantly, these results reveal how the basic chemical makeup of asbestos and crocidolite asbestos can change phenotypic expression by directly altering the local concentration of an important signaling molecule (i.e., NO).

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Figure 3. Western blot analysis demonstrating crocidolite-induced alterations in NO-mediated posttranslational regulation of HIF-1α and p53. A, cells were plated into six-well culture plates, grown to 85% confluency, and serum-starved overnight. They were treated with the NO donor Sper/NO for 2 hours and harvested. Decomposed Sper/NO (100 μmol/L) was used as a negative control at 2 hours (data not shown). Steady-state NO concentrations ([NO]ss nmol/L) were determined from 100-μL sample aliquots of medium withdrawn from the culture plate by gas-tight syringe without agitation and were analyzed by chemiluminescence (graph). Columns, mean (n = 3); bars, SE. B, JMN cells were plated into six-well culture plates, grown to 85% confluency, and serum-starved overnight. The cells were treated for 2 hours ± Sper/NO and ± crocidolite (1–100 μg/cm², 80 μg shown) and immediately harvested for Western blot analysis. C, MCF-7 cells were plated into six-well culture plates, grown to 85% confluency, and serum-starved overnight. The cells were treated for 2 hours ± Sper/NO and ± crocidolite and were immediately harvested. Representative immunoblots (n > 3).
and physical properties of asbestos are sufficient to significantly influence cellular phenotype.

An extensive body of work has established that both inducible NOS and manganese superoxide dismutase (MnSOD) expression are high in malignant mesotheliomas and metastatic pleural tumors compared with nonneoplastic mesothelium and healthy pleural tissues (18, 19). Furthermore, alveolar macrophages from asbestos-exposed rats generated significantly greater nitrite than did macrophages from sham-exposed rats (14). It follows that this phenotype should favor the formation of NO2 and H2O2 from inducible NOS and MnSOD, respectively. We judge this to be especially important because our data indicate that when asbestos is present, H2O2 is an ideal substrate for both NO consumption to NO2 and the subsequent formation of 3-nitrotyrosine.

Examining the effects of crocidolite on HIF-1α and p53 regulation by NO was critical because not only is p53 up-regulated in vivo after asbestos exposure, but both HIF-1α and p53 genes expression are increased in malignant mesotheliomas (20). These studies on posttranslational protein regulation by NO revealed the striking effect crocidolite had on altering these processes. As such, this suggests that some of the phenotypic transformations in gene expression and protein regulation as seen in malignant mesotheliomas may simply be due to the disruption of normal levels of signaling molecules (i.e., NO). Because greater concentrations of NO are necessary to induce p53 P-Ser-15 than are required to cause HIF-1α accumulation (7), it is conceivable that asbestos may sufficiently reduce local NO concentrations to transform the cellular growth phenotype from inhibitory (p53 P-Ser-15) to proliferative (HIF-1α). Notably, this work suggests that the etiology of malignant mesothelioma may stem from the ability of asbestos to generate free radicals and disrupt NO-mediated host regulatory mechanisms.

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