Crocidolite Activates NF-κB and MIP-2 Gene Expression in Rat Alveolar Epithelial Cells. Role of Mitochondrial-Derived Oxidants

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Nuclear factor kappa B (NF-κB) is a transcription factor that regulates expression of several genes coding for inflammatory and immunoregulatory proteins including the neutrophil chemotactic cytokine MIP-2. In previous studies we found that crocidolite asbestos activates the nuclear translocation of NF-κB as well as MIP-2 gene expression in rat alveolar type II cells. Here we report that both crocidolite-induced NF-κB activation of MIP-2 gene expression can be attenuated by the antioxidant tetramethylthiourea, suggesting the dependence of these responses on oxidative stress. Crocidolite exposure of RLE-6TN cells also increased production of H₂O₂, a response that was inhibited by the mitochondrial respiratory chain inhibitor thienoyltrifluoroacetone (TTF-A). TTF-A treatment of RLE-6TN cells also inhibited crocidolite-induced nuclear translocation of NF-κB and MIP-2 gene expression. These results indicate crocidolite exposure of rat alveolar type II cells results in increased production of mitochondrial-derived hydrogen peroxide and that mitochondrial-derived oxidants contribute to crocidolite activation of NF-κB and increases in MIP-2 gene expression. — Environ Health Perspect 106(Suppl 5):1171–1174 (1998).

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Introduction

Occupational exposure to crocidolite asbestos has been associated with development of chronic interstitial lung disease, lung cancer, and mesothelioma (1–3). As with a number of pneumotoxic agents, the alterations in lung structure and function arising from crocidolite exposure result, at least in part, from the recruitment and activation of inflammatory cells. In this respect, studies using animal models of asbestosis have demonstrated that pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1 contribute to the crocidolite-induced pulmonary inflammation (4–6). More recently, in vitro and in vivo studies have demonstrated that potent leukocyte chemotactic cytokines known as chemokines are mediators of crocidolite-induced inflammation (4,7,8).

Macrophage inflammatory protein 2 (MIP-2) is an 8-kDa, heparin-binding protein first identified as a product of endotoxin-stimulated mouse macrophages (9). MIP-2 is a member of the chemokine family and is selectively chemotactic for neutrophils and mitogenic for alveolar epithelial cells (10). Murine MIP-2 is functionally and structurally related to the human gro chemokines (10). Exposure of rats to a variety of particles including crocidolite and quartz under conditions eliciting pulmonary inflammation results in increased MIP-2 expression in the lung (5,8,10). Sources of MIP-2 in the rat lung after particle exposure include both the alveolar macrophage and lung epithelial cells (8). Regarding the latter, in vitro studies have demonstrated that crocidolite can directly activate alveolar type II cells to express the chemokines MIP-2 and IL-8 (8,11).

Although the mechanisms by which crocidolite activates rat lung epithelial cell MIP-2 production are unknown, expression of the MIP-2 gene is regulated by the transcription factor nuclear factor kappa B (NF-κB) (12). Studies in several different systems indicate the signaling pathways regulating NF-κB involve an oxidant component, although the nature of the oxidant and the mechanism by which it is coupled to the signaling cascade remains undefined (13). To better understand the mechanisms by which crocidolite activates MIP-2 gene expression and activation of NF-κB, the present studies investigated the contribution of oxidative stress to crocidolite-induced activation of NF-κB and MIP-2 gene expression; and the potential source of oxidants contributing to this response.

Methods

Culture and in Vitro Exposure of Rat Alveolar Epithelial Cells

Epithelial cell responses to particles were characterized using the rat alveolar type II cell line, RLE-6TN (14). This cell line retains characteristics of alveolar type II cells, including expression of chemokines after exposure to various agonists (8,14). RLE-6TN cells were maintained in 25-cm² tissue culture flasks and grown in RuE media (BRFF, Ijamsville, MD). The effects of the oxygen radical scavenger tetramethylthiourea (TMTU) or the mitochondrial respiratory chain inhibitor thienoyltrifluoroacetone (TTF-A) on in vitro crocidolite activation of nuclear translocation of NF-κB and MIP-2 gene expression were examined using confluent cultures of RLE-6TN cells exposed under serum-free conditions to crocidolite (median diameter ± GSD (geometric standard deviation) ) = 0.28 ± 1.8; length [% < 10 µm] = 23; surface area = 3.2 m²/g) at 10 µg particles/cm² of the culture dish for a period of 6 hr. A dose of 10 µg/cm² was used because previous studies in our laboratory demonstrated this dose was...
effective at activating these responses in RLE-6TN cells (8,15).

Electrophoretic Mobility Shift Assay
The electrophoretic mobility shift assay was used to characterize NF-κB-binding activity as described previously (16). Briefly, RLE-6TN cells were rinsed 2 times with cold phosphate-buffered saline, pH 7.2, and the nuclear extracts collected, as described by Staal et al. (17). Cells were lysed in hypotonic buffer, 0.6% Nonidet P-40 was added, and lysates were vortexed for 15 sec. Nuclei were pelleted by centrifugation at 4°C. Nuclear protein extracts (4 μg) were incubated for 20 min in DNA-binding buffer (40 nM HEPES buffer, 4% Ficoll 400, 200 ng polyI:polyC per μl, 1 mM MgCl₂, 0.1 mM dithiothreitol, and 0.175 pmol 32P-end-labeled double-stranded oligonucleotide containing a consensus NF-κB site (Promega, Madison, WI)). Samples were loaded onto a 5% polyacrylamide gel and electrophoresed in 0.25×Tris borate–EDTA buffer for 2 hr at 120 V. Gels were dried and visualized by exposure to Kodak X-Omatfilm.

Reverse Transcriptase–Polymerase Chain Reaction Analysis of Chemokine Expression
MIP-2 mRNA transcript levels were assessed by polymerase chain reaction (PCR) amplification of the MIP-2 cDNA, as described in detail elsewhere (10). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was evaluated as an internal control. The primers were designed from the published sequences for MIP-2 (18) and GAPDH (19) and were as follows: MIP-2: 5'-GGCACATCAGG TACGATCCAG-3'; 5'-ACCTGCCCCAAGGGTGTACCTC-3'; GAPDH: 5'-CAGGATGGTCAGCTGACATC-3'; 5'-GGTGCGGTGTGAACGGATTG-3'.

PCR reactions were overlaid with mineral oil and amplification carried out between 22 to 30 cycles of denaturation at 94°C for 1 min, oligo annealing at 55°C for 1 min, and extension at 72°C for 2 min. Reactions were electrophoresed in 1.5% agarose gels containing ethidium bromide in Tris acetate–EDTA buffer to visualize the MIP-2 and GAPDH PCR products.

Measurement of H₂O₂
To characterize the effects of crocidolite on production of H₂O₂, RLE-6TN cells were cultured at 1×10⁶ cells/well in 96-well tissue culture plates (Corning, Corning, NY) and exposed to crocidolite at 10 μg/cm² with and without catalase (100 U/ml; Sigma Chemical Co., St. Louis, MO) or the mitochondrial respiratory chain inhibitor TTFA at 200 μM. Hydrogen peroxide in RLE-6TN cell conditioned media was determined after 6 hr of crocidolite exposure based on the horseradish peroxidase-dependent conversion of phenol red by H₂O₂ into a compound with absorbance at 600 nm, as described by Ding et al. (20). Briefly, 100 μl phenol red solution (140 mM NaCl, 10 mM KH₂PO₄, 5.5 mM dextrose, 0.56 mM phenol red) (Sigma) containing 6 U/ml type VI horseradish peroxidase (Sigma) was added to cell cultures. After 6 hr at 37°C, 10 μl 1 N NaOH was added and absorbance at 600 nm was determined using an EL311 Biotek Microplate Reader (Biotek Instruments, Winooski, VT). H₂O₂ concentrations were calculated from a standard curve.

Statistical Analysis
Data for H₂O₂ production were analyzed by ANOVA, with group differences determined using the Newman–Keuls test (21).
Discussion

The transcription factor NF-κB can be activated by a variety of pathologic stimuli and regulates the transcription of several genes that contribute to inflammatory processes (22). Previous studies have demonstrated that crocidolite can increase nuclear translocation of NF-κB in several cellular targets of asbestos-induced lung disease, including hamster tracheal epithelial cells, rat pleural mesothelial cells, rat alveolar type II cells, and a human lung carcinoma cell line (15,16,23). The present studies confirm and extend previous observations by demonstrating that crocidolite exposure of the rat alveolar type II cell line RLE-6TN results in activation of NF-κB, a response associated with increased cellular production of H₂O₂ and inhibited by antioxidants. In addition, this is the first study to show that, like NF-κB, the chemokine MIP-2 is regulated by oxidants and to provide evidence that the source of oxidants contributing to crocidolite activation of and MIP-2 gene expression in rat alveolar type II cells is the mitochondria.

MIP-2 is a key mediator of neutrophil recruitment in the rat lung and plays a role in pulmonary inflammation evoked by a variety of agents, including pneumotoxic particles such as crocidolite and quartz, high lung doses of relatively innocuous materials such as titanium dioxide and carbon black, and the oxidant air pollutant ozone (5,8,24). Recent studies indicate that some particles, including crocidolite, can directly activate MIP-2 gene transcription in rat alveolar type II cells and expression of IL-8 mRNA in the human lung carcinoma cell line A549 (8,23). Analysis of the proximal promoter region of the MIP-2 gene revealed that it contains a κB site that regulates endotoxin activation of MIP-2 gene transcription in macrophages (12). Results of the present studies demonstrate crocidolite-induced expression of MIP-2 in epithelial cells is associated with NF-κB activation and is sensitive to the same factors that inhibit nuclear translocation of NF-κB, suggesting that like endotoxin activation of MIP-2 gene transcription in macrophages, NF-κB contributes to crocidolite-induced MIP-2 gene transcription in epithelial cells. There is now considerable evidence that reactive oxygen species influence the activation of NF-κB (13). Regarding crocidolite activation of RLE-6TN cells, our demonstration that the nuclear translocation of NF-κB and increased MIP-2 mRNA expression can be inhibited by the oxygen radical scavenger TMTU indicates oxidants play a role in these responses. This observation is consistent with recent studies demonstrating that crocidolite-induced increases in nuclear NF-κB in hamster tracheal epithelial cells can be attenuated by treatment with the sulfhydryl reagent N-acetylcysteine (16). One mechanism by which crocidolite could produce an oxidative stress on cells is through redox reactions catalyzed by metals on the surface of the fibers (25). Alternatively, oxidants could be produced as a result of the fibers stimulating production of cell-derived oxidants. As reviewed by Kamp et al. (26), macrophages and neutrophils respond to crocidolite with production of superoxide anion radicals and H₂O₂. The observation that crocidolite exposure increased H₂O₂ release by RLE-6TN cells indicates that, like macrophages and neutrophils, crocidolite can stimulate production of reactive oxygen species by rat alveolar epithelial cells.

There are several potential sources of oxidants within cells, including lipoxigenases, xanthine oxidase, cytochrome P450, and mitochondria. Regarding the latter, mitochondria are known to be sources of H₂O₂ (27,28). At both complex I (NADH dehydrogenase) and complex III of the respiratory chain, there can occur a single electron reduction of molecular oxygen resulting in the formation of superoxide anion radicals (29,30). Through either its spontaneous dismutation or the action of manganese superoxide dismutase, superoxide anion radicals in the mitochondria can be converted to H₂O₂. Our findings with TTFα, an inhibitor of electron transfer from complex II to complex III, which attenuates superoxide anion radical formation by mitochondria, indicate the mitochondrial respiratory chain is a source of H₂O₂ in crocidolite-exposed rat alveolar epithelial cells. Additionally, the observation that TTFα treatment also inhibited crocidolite activation of NF-κB and MIP-2 gene expression provides evidence that mitochondrial-derived oxidants are a part of a signaling pathway activated by this fiber. In this respect, the cytokine TNF-α, a potent activator of NF-κB, increases oxidant production by mitochondria (31). Similar to our observations, studies using the mitochondrial respiratory chain inhibitor rotenone have demonstrated that mitochondrial-derived oxidants contribute to TNF-α-induced nuclear translocation of NF-κB (32), suggesting there are common aspects in the signaling pathways regulating NF-κB activation in cells exposed to crocidolite and TNF-α.

In summary, here we report that crocidolite exposure of the rat alveolar type II cell line increases nuclear translocation of the transcription factor NF-κB and expression of mRNA for the neutrophil chemotactic chemokine MIP-2. Both the NF-κB and the MIP-2 responses could be attenuated by the antioxidant TMTU, indicating their dependence on oxidative stress. Exposure of RLE-6TN cells increased production of...
H$_2$O$_2$ by the epithelial cells. The source of the H$_2$O$_2$ after crocidolite exposure appears to be the mitochondria, as treatment with the mitochondrial respiratory chain inhibitor TTFA inhibited the H$_2$O$_2$ response. Last, TTFA attenuated crocidolite activation of NF-κB and MIP-2 gene expression in the RLE-6TN cells, indicating that mitochondria-derived oxidants play a role in the signaling pathway, resulting in increased nuclear translocation of NF-κB and MIP-2 gene transcription.

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