

Possible Role of Lipid Peroxidation in the Induction of NF- κ B and AP-1 in RFL-6 Cells by Crocidolite Asbestos: Evidence following Protection by Vitamin E

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Asbestos fibers cause persistent induction of the oxidative stress sensitive transcription factors nuclear factor kappa-B (NF- κ B) and activator protein-1 (AP-1) in mammalian cells. These transcription factors play an important role in the regulation of cellular activity. Lipid peroxidation, mediated by reactive oxygen species, is thought to be a possible mechanism in the pathogenicity of asbestos fibers. These studies were designed to determine if crocidolite asbestos-induced lipid peroxidation plays a role in the mechanism of formation of NF- κ B and AP-1. Treatment of a rat lung fibroblast cell line (RFL-6) with crocidolite asbestos in the presence and absence of the membrane antioxidant vitamin E decreased the levels of crocidolite-induced AP-1 and NF- κ B to background levels. Preincubation of RFL-6 cells with 5,8,11,14-eicosatetraenoic acid, an inhibitor of arachidonic acid metabolism, prior to exposure to crocidolite, abrogated crocidolite-induced NF- κ B DNA-binding activity to background levels. Coincubation with indomethacin, a cyclooxygenase inhibitor, had no effect on NF- κ B DNA-binding activity induced by crocidolite. However, nordihydroguaiaretic acid, a lipoxygenase inhibitor, decreased levels of NF- κ B to background levels. This would suggest that lipoxygenase metabolites of arachidonic acid, produced following lipid peroxidation, are involved in the cellular signalling events leading to NF- κ B transcription factor induction by asbestos. — *Environ Health Perspect* 105(Suppl 5):1127–1130 (1997)

Key words: lipid peroxidation, asbestos, NF- κ B, AP-1

Introduction

Occupational exposure to asbestos fibers has been linked to the development of pulmonary fibrosis, bronchogenic carcinomas, and malignant mesotheliomas of the pleura and peritoneum (1,2). The underlying mechanisms by which asbestos induces respiratory malignancies are unclear. In addition to size and fiber durability being important in fiber pathogenicity, asbestos fibers can generate reactive oxygen species (ROS) from redox reactions catalyzed on the fiber surface or from incomplete phagocytosis of the fibers. ROS can react with DNA, which can cause DNA strand breaks (3,4) and base modifications (5,6)

as well as induce cellular oxidative stress in which antioxidant defenses are compromised. Recent studies by Heintz et al. (7) have shown that both crocidolite and chrysotile asbestos cause dose dependent and persistent increases in expression of *c-fos* and *c-jun* in rat pleural mesothelial cells and *c-jun* in hamster tracheal epithelial cells. Both *c-fos* and *c-jun* are immediate early response genes associated with transition of the cells from the G1 stage of the cell cycle to S phase (8), and their induction may provide the molecular switch for cell proliferation. Induction of *c-fos* and *c-jun* is accompanied by increased binding

of the transcription factor activator protein-1 (AP-1), a homodimeric (Jun–Jun) or heterodimeric (Fos–Jun) protein complex to DNA. In addition, recent studies have shown that crocidolite asbestos induces an increase in nuclear factor kappa B (NF- κ B) DNA binding and NF- κ B-dependent genes such as *c-myc* (9).

ROS can initiate peroxidation of membrane lipids and there is now considerable evidence that products of lipid peroxidation are involved in carcinogenesis (10). Studies in our laboratory (11) have shown that mineral fibers cause time- and dose-dependent induction of lipid peroxidation, as evidenced by increases in thiobarbituric acid reactive products, in a rat lung fibroblast cell line (RFL-6). Additionally, certain end products of this process bind to cellular DNA and this may be a mechanism in the genotoxic action of asbestos fibers (11). Recent studies have shown that H₂O₂-induced *c-fos* expression in rodent smooth muscle cells is mediated by products of lipid peroxidation, arachidonic acid (AA) in particular, and from metabolism of AA via the lipoxygenase pathway (12). Other studies have shown that fecapentaene-12, a potent mutagen in the colon, causes oxidative damage in HeLa cells (13) and induces *c-jun* via the cyclooxygenase component of this pathway (SM Plummer, personal communication). The involvement of the lipoxygenases and leukotriene biosynthesis or cyclooxygenases and prostaglandin biosynthesis in the signaling pathway, which lead to the induction of transcription factors by asbestos, has not been assessed. In this study we assessed the role of lipid peroxidation in asbestos-induced DNA binding of transcription factors AP-1 and NF- κ B by incubating RFL-6 cells with crocidolite asbestos in the presence and absence of vitamin E, an inhibitor of lipid peroxidation (14). In addition, further mechanistic studies in RFL-6 cells on crocidolite-mediated NF- κ B induction were undertaken by preincubation of cells with an inhibitor of AA metabolism, 5,8,11,14-eicosatetraenoic acid (ETYA) (15), or with potent inhibitors of cyclooxygenase and lipoxygenase pathways, indomethacin (16) and nordihydroguaiaretic acid (NDGA) (17), respectively, prior to asbestos exposure. We report here that lipoxygenase metabolites of AA, produced after lipid peroxidation, are involved in the induction of the transcription factor NF- κ B by asbestos.

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Abbreviations used: AA, arachidonic acid; AP-1, activator protein-1; ETYA, 5,8,11,14-eicosatetraenoic acid; 4-HNE, 4-hydroxynonenal; NDGA, nordihydroguaiaretic acid; NF- κ B, nuclear factor kappa B; RFL-6, rat lung fibroblast cell line; ROS, reactive oxygen species.

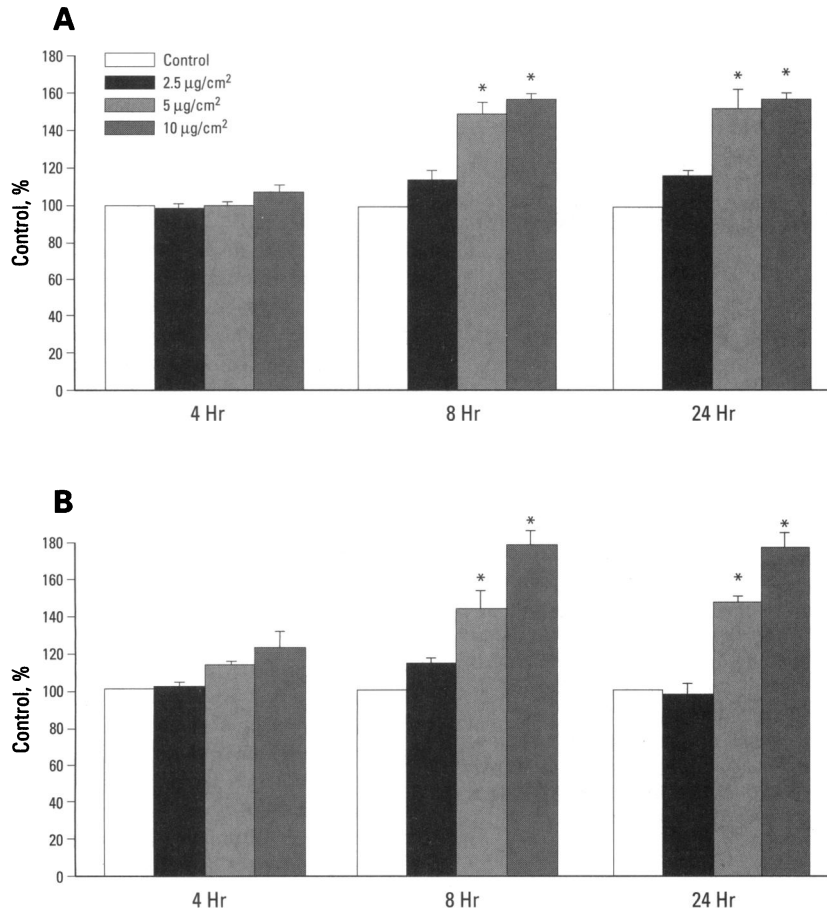


Figure 1. Effect of crocidolite asbestos on the induction of transcription factors (A) AP-1 and (B) NF- κ B in RFL-6 cells. The results are mean \pm SEM for each treatment and time point ($n=3$). *, differ significantly from controls ($p<0.05$).

Methods

Reference samples of Union Internationale Contre le Cancer processed crocidolite fiber were a kind gift from R.C. Brown (MRC Toxicology Unit, Leicester University, Leicester, U.K.). All other chemicals were purchased from Aldrich (Gillingham, Dorset, U.K.) or Sigma Chemical Co. (Poole, Dorset, U.K.) unless otherwise stated.

Cell Culture and Exposure to Test Agents

A rat lung fibroblast cell line (RFL-6; European Collection of Animal Cell Cultures, Porton Down, Wiltshire, U.K.) was grown in Hams F-12 medium containing L-glutamine (Gibco, Paisley, U.K.) supplemented with penicillin (50 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), nonessential amino acids (1%; Gibco) and fetal bovine serum (10%; Gibco). Cells were grown to confluence and 24 hr prior to addition of crocidolite asbestos and other agents, the

growth medium was replaced with medium containing 2% serum. RFL-6 cells were incubated initially with crocidolite fibers for 4, 8, and 24 hr at 37°C at final concentrations of 2.5, 5, and 10 $\mu\text{g}/\text{cm}^2$. Additional treatments were set up with crocidolite fibers (5 and/or 10 $\mu\text{g}/\text{cm}^2$) in the presence and absence of vitamin E (0.5 and 2 mM), indomethacin (50 and 100 μM), NDGA (10 and 50 μM), or ETYA (5 μM) for 8 hr at 37°C.

Gel Mobility Shift Assays for AP-1 and NF- κ B DNA Binding Activities

One nanogram of ^{32}P end-labeled oligonucleotide containing the AP-1 or NF- κ B consensus DNA binding sequence was incubated with 2 μg whole cell protein extract prepared as described by Staal et al. (18). The protein-DNA complexes were resolved by electrophoresis in nondenaturing 4% polyacrylamide gels and the retarded bands were quantified with a

phosphoimager (Molecular Dynamics, Chesham, Bucks, U.K.).

Results

Crocidolite asbestos stimulated both NF- κ B and AP-1 transcription factor expression in both a time- and dose-dependent manner in RFL-6 cells, with maximum induction at 8 and 24 hr at 5 and 10 $\mu\text{g}/\text{cm}^2$ ($p<0.05$) (Figure 1). To examine whether lipid peroxidation mediated by asbestos is involved in the induction of transcription factors AP-1 and NF- κ B, crocidolite asbestos was added to RFL-6 cells in the presence and absence of the membrane antioxidant vitamin E. The increase in both AP-1 and NF- κ B by crocidolite was ameliorated in the presence of vitamin E at both concentrations ($p<0.05$) (Figure 2).

To examine the involvement of AA metabolism in NF- κ B transcription factor induction by asbestos, we incubated RFL-6 cells with crocidolite in the presence and absence of ETYA, which is an inhibitor of AA metabolism. The levels of NF- κ B DNA binding by crocidolite in the presence of ETYA were reduced to background levels (Table 1). To investigate further the involvement AA metabolism in NF- κ B induction by asbestos, specifically either the cyclooxygenase or lipoxygenase components of this metabolic pathway, we incubated RFL-6 cells with crocidolite in the presence and absence of indomethacin, an inhibitor of the cyclooxygenase pathway ($\text{IC}_{50}=0.1 \mu\text{M}$), or NDGA, an inhibitor of the lipoxygenase pathway ($\text{IC}_{50}=0.2 \mu\text{M}$ for 5-lipoxygenase and 30 μM for 12- and 15-lipoxygenases). Treatment of cells with crocidolite in the presence of indomethacin did not alter NF- κ B DNA binding in RFL-6 cells (Figure 3). However, in the presence of NDGA, the levels of NF- κ B DNA binding induced by crocidolite were significantly reduced ($p<0.05$) compared to levels seen with crocidolite alone (Figure 4).

Discussion

There is strong evidence that signaling pathways are involved in the enhanced expression of oxidative stress-inducible genes such as *c-fos*, *c-jun*, and transcription factors AP-1 and NF- κ B (19,20). There is also data to support the initiation of these signaling events at the plasma membrane (21,22) that involve kinase cascades (21) and redox regulation (23). Because cellular membranes are an important target for asbestos-mediated ROS production, we analyzed whether damage to membrane lipids is

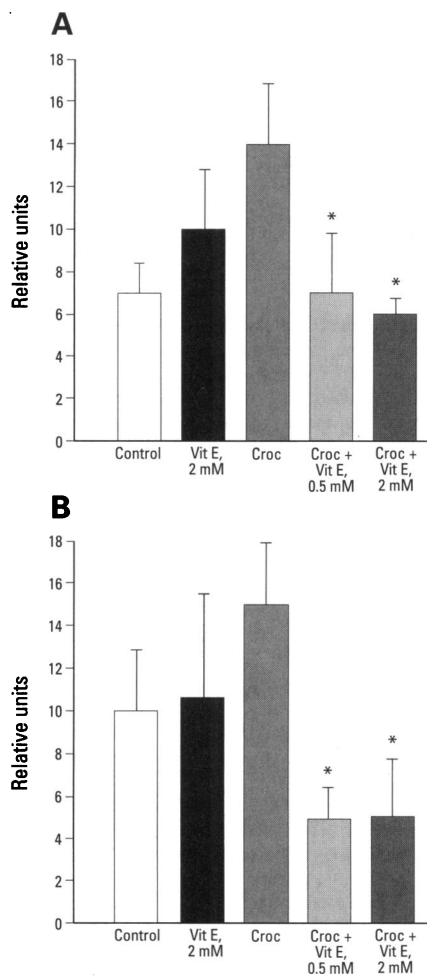


Figure 2. Effect of vitamin E (Vit E) on crocidolite (Croc; 5 μg/cm²)-mediated induction of (A) NF-κB and (B) AP-1 DNA binding activities in RFL-6 cells. The results are shown as mean ± SEM for each treatment (n=3). *, differ significantly from treatments with crocidolite alone (p<0.02).

Table 1. Effect of ETYA on crocidolite-induced NF-κB DNA binding activity in RFL-6 cells.

Treatment	NF-κB DNA binding, relative units
Control	7.11 ± 0.55
Crocidolite (5 μg/cm ²)	10.69 ± 0.16
Crocidolite (5 μg/cm ²) + ETYA (5 μM)	7.54 ± 0.04*

Results are mean ± SEM for each treatment (n=3). *, differs significantly from treatments with crocidolite alone (p<0.05).

involved in the induction of the oxidative stress-sensitive transcription factors AP-1 and NF-κB. Our results indicate that peroxidation of cellular membranes is important for crocidolite asbestos induction of AP-1 and NF-κB. When cells were treated with

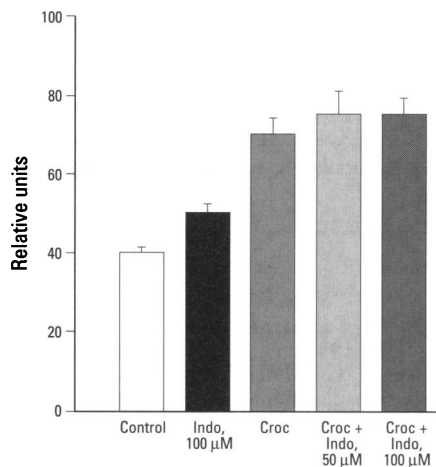


Figure 3. Effect of indomethacin (Indo) on crocidolite (5 μg/cm²)-mediated induction of NF-κB DNA binding in RFL-6 cells. The results are shown as mean ± SEM for each treatment (n=3).

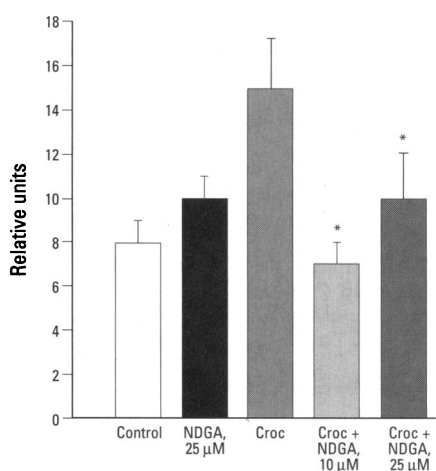


Figure 4. Effect of nordihydroguaiaretic acid on crocidolite (5 μg/cm²)-mediated induction of NF-κB DNA binding in RFL-6 cells. The results are the mean ± SEM for each treatment (n=3). *, differ significantly from treatments with crocidolite alone (p<0.05).

crocidolite in the presence of the membrane antioxidant vitamin E, the levels of DNA binding of AP-1 and NF-κB were reduced to background levels. The lipophilic antioxidant vitamin E inhibits lipid peroxidation by scavenging lipid radicals and thereby halting the propagation of the process and preventing accumulation of lipid hydroperoxides that subsequently decompose to a wide range of end products, such as malondialdehyde, hexanal, and 4-hydroxynonenal (4-HNE) (24). 4-HNE can also mediate the activation of enzymes involved in signal transduction, such as adenylate cyclase and phosphatidy inositol

4,5-biphosphate-phospholipase C in membrane preparations (25,26) and phospholipase D in cells (27). In addition, endogenous 4-HNE has the potential to activate these enzymes within these cells during oxidative stress because this and other aldehydes have half-lives longer than most ROS and can diffuse within or out of the cell (28). Besides the well characterized function of vitamin E as an antioxidant, alternative roles such as that of a membrane stabilizer and regulator of membrane fluidity have been proposed (29). In addition, vitamin E is an inhibitor of cellular 5-lipoxygenase activity (30), thus preventing breakdown of AA into active metabolites.

This study investigated whether asbestos-mediated induction of the transcription factor NF-κB involves metabolites of AA as second messengers via the cyclooxygenase or lipoxygenase pathway. In enzymatic lipid peroxidation, oxidized fatty acids such as AA are released preferentially by phospholipase A₂ from membrane lipids. Regardless of the source of its release, AA is highly relevant to signal transduction within the cell because it is a substrate for synthesis of eicosanoids, namely prostaglandins and leukotrienes, by cyclooxygenases and lipoxygenases, respectively. Little is known about the role of AA or its metabolites in asbestos-mediated gene transcription, but in recent studies, the induction of *c-fos* gene expression with agents such as tumor necrosis factor (31) or H₂O₂ (12) was mediated by the conversion of AA to a metabolite(s) via the lipoxygenase pathway. In these studies (12,31) cyclooxygenase inhibitors had no effect on the modulation of mRNA levels of *c-fos*. We showed for the first time that AA metabolites are involved in the cellular signaling events leading to NF-κB induction by asbestos. In the present study, asbestos treatment of cells in the presence of ETYA, an inhibitor of AA metabolism (15), reduced NF-κB DNA binding to basal levels. In addition, with the use of inhibitors of the cyclooxygenase and lipoxygenase pathways, namely indomethacin (16) and NDGA (17), respectively, we demonstrated that lipoxygenase metabolites are involved in NF-κB induction by asbestos. In these studies, asbestos treatment of cells in the presence of NDGA reduced NF-κB DNA binding to basal levels. In addition, the concentration of NDGA (10 μM) that reduced transcription factor induction would suggest that it is the 5-lipoxygenase pathway that is being inhibited (12). In

contrast, indomethacin had no effect on the crocidolite response in these cells.

In summary, this study has shown that lipid peroxidation is involved in asbestos-mediated cellular signaling events, which lead to AP-1 and NF- κ B transcription

factor induction, after inhibition with the membrane antioxidant vitamin E. In addition, our results suggest that when RFL-6 cells are treated with crocidolite asbestos, AA is released following lipid peroxidation and processed via the lipoxygenase pathway

to a metabolite or metabolites required for crocidolite-mediated NF- κ B induction. Studies are in progress to determine which lipoxygenase product or products are involved in the cellular signaling pathways mediated by asbestos.

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