Antioxidants Prevent the RhoA Inhibition Evoked by Crocidolite Asbestos in Human Mesothelial and Mesothelioma Cells

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Asbestos is a naturally occurring fibrous silicate, whose inhalation is highly related to the risk of developing malignant mesothelioma (MM), and crocidolite is one of its most oncogenic types. The mechanism by which asbestos may cause MM is unclear. We have previously observed that crocidolite in human MM (HMM) cells induces NF-κB activation and stimulates the synthesis of nitric oxide by inhibiting the RhoA signaling pathway. In primary human mesothelial cells (HMCs) and HMM cells exposed to crocidolite asbestos, coincubated or not with antioxidants, we evaluated cytotoxicity and oxidative stress induction (lipid peroxidation) and the effect of asbestos on the RhoA signaling pathway (RhoA GTP binding, Rho kinase activity, RhoA prenylation, hydroxy-3-methylglutharyl-CoA reductase activity). In this paper we show that the reactive oxygen species generated by the incubation of crocidolite with primary HMCs and three HMM cell lines mediate the inhibition of 3-hydroxy-3-methylglutharyl-CoA reductase (HMGCR). The coincubation of HMCs and HMM cells with crocidolite together with antioxidants, such as Tempol, Mn-porphyrin, and the association of superoxide dismutase and catalase, prevented the cytotoxicity and lipoperoxidation caused by crocidolite alone as well as the decrease of HMGCR activity and restored the RhoA/RhoA-dependent kinase activity and the RhoA prenylation. The same effect was observed when the oxidizing agent menadione was administrated to the cells in place of crocidolite. Such a mechanism could at least partly explain the effects exerted by crocidolite fibers in mesothelial cells.

Keywords: crocidolite; mesothelioma; antioxidants; reactive oxygen species; RhoA

CLINICAL RELEVANCE

Antioxidant prevention of crocidolite asbestos-mediated reactive oxygen species production, by restoring RhoA prenylation, is important not only in controlling asbestos oxidative stress but also should be related to the cellular transformation into a malignant mesothelioma.

Exposure to asbestos is associated with pulmonary fibrosis and tumor diseases such as lung cancer and malignant mesothelioma (MM). Asbestos is a generic term indicating a group of fibrous minerals that can be subdivided into two major groups: amphiboles and serpentine (1). The amphibole fibers include crocidolite, which is often considered the most oncogenic type of asbestos (2). Although a causative linkage between asbestos exposure and lung diseases has been well established epidemiologically, the molecular mechanisms by which crocidolite or other asbestos forms induce these pathologies are poorly understood. However, accumulating evidence has established that reactive oxygen species (ROS) and reactive nitrogen species are important second messengers of asbestos toxicity (3, 4). The mechanisms responsible for asbestos-induced free radical generation are in part caused by reactions occurring at the surface of mineral dusts, by the activation of alveolar macrophages or neutrophils attempting to take up the fibers, and by the mitochondrial dysfunction in target cells (5). Furthermore, we have previously observed that crocidolite inhibits the pentose phosphate oxidative pathway and glucose 6-phosphate dehydrogenase activity in human lung epithelial cells (6), providing a further reason for ROS generation (i.e., a decrease of one of the main antioxidant pathways of the cell). Asbestos, by stimulating ROS production, may modulate different redox-sensitive signaling pathways, such as NF-κB and activating protein-1 (7).

In human MM (HMM) cells, we have previously demonstrated that crocidolite exposure down-regulates the ability of the small G protein RhoA (Ras homologous small GTPase A) to bind GTP and the RhoA-dependent kinase (ROCK) activity. In that work, we correlated the crocidolite-induced RhoA inhibition with the activation of the IkB (inhibitor kB) α kinase and the Akt/PKB (protein kinase B) signaling pathway, thus leading to NF-κB translocation and to nitric oxide (NO) radical generation (8). Because mevalonate, the product of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), reversed all the effects of crocidolite except the crocidolite-induced accumulation of the products of lipid peroxidation (8), we assumed that the inhibition was dependent on an oxidative impairment at the level of the rate-limiting enzyme HMGCR or of an upstream step, but we did not provide an explanation for the mechanism.

HMGCR is sensitive to redox regulation, although the data concerning this enzyme are few. It has been shown that each of the four identical subunits of *Pseudomonas mevalonii* HMGCR contains two cysteine residues (cys156 and cys296), both of which are accessible to modification by oxidant reagents (9). Rat liver microsomal HMGCR has been demonstrated to be extremely sensitive to oxidative inactivation, which involves the formation of an intramolecular S-S disulfide bond inactivating the enzyme (10, 11).

We investigated whether, in human mesothelial cells (HMCs) and MM cells, the crocidolite-induced RhoA inhibition might be ascribed to the increased generation of ROS and to the subsequent oxidative inhibition of HMGCR, thus adding a new possible link between the oxidative stress evoked by asbestos exposure and the development of MM.

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MATERIALS AND METHODS

FBS and HAM'S F-12 nutrient mixture medium were supplied by BioWhittaker (Verviers, Belgium). Plasticware for cell culture was from Falcon (Becton Dickinson, Bedford, MA). Electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA). The protein content was assessed with the BCA kit from Pierce (Rockford, IL). Mn-porphyrin [manganese(III)meso-tetra-(4-N-methylpyridinium)porphyrin] and other reagents, when not otherwise specified, were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells

Primary HMCs and HMM cell lines MM98, OC99, and GF99 were obtained from the biobank of the Hospital of Alessandria (Pathology Unit). HMCs were isolated from three patients with no history of malignant disease. HMM cell lines were obtained from the pleural effusions of three patients with histologically confirmed MM, as previously described (12).

Asbestos Fibers

UICC (Union International Contre le Cancer) crocidolite fibers were sonicated (100 W, 30 s) (Labsonic Sonicator; Sartorius Stedim Biotech S.A., Aubagne, France) before incubation with cell cultures to dissociate fiber bundles and allow better suspension in the culture medium. The involvement of endotoxins, known contaminants of many particulates, was ruled out by coincubating the endotoxin inhibitor polymixin B (10 μg/ml) on cell cultures with crocidolite; no change in asbestos-related effects was observed in the presence of polymixin B (data not shown).

Extracellular Lactate Dehydrogenase Activity

Lactate dehydrogenase (LDH) activity was measured in the extracellular medium and in the cell lysate as previously described (14).

Measurement of Lipid Peroxidation by 8-Isoprostane Assay

The levels of free 8-epi-PGF2α were measured using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) (15). Briefly, 50 μl of standard or culture supernatant samples were placed in a 96-well plate precoated with mouse monoclonal antibody after purification. The absorbance of plates was read at 412 nm, and the 8-epi-PGF2α levels were calculated according to a calibration curve.

RhoA-GTP Binding Assay

Rho-GTP binding activity was measured using the G-LISA Kit (Cytoskeleton, tebu-bio, Milan, Italy) as previously described (8).

ROCK Assay

ROCK activity was measured as previously described (8).

Analysis of RhoA Prenylation

Cells were separated, and proteins were analyzed by Western blotting as previously described (8). As control for equal loading of samples, the expression of actin (Santa Cruz Biotechnology, Santa Cruz, CA) in whole lysate and the content of porin (Calbiochem, San Diego, CA) in detergent-rich fractions were analyzed.

Measurement of HMGCR Activity

The HMGCR activity was assayed according to Philipp and colleagues (16), with modifications as previously described (17). Cell lysates and microsomal fractions were supplemented with 10 mM dithiothreitol. HMGCR activity was expressed as nmol HMG-CoA/mg cell proteins.

Immunofluorescence Microscopy

Cells were seeded on glass coverslips and loaded with the actin phalloidin-tetramethylrhodamine B isothiocyanate and the nuclear 4′,6-diamidino-2-phenylindole dihydrochloride dye and examined with a DC100 Leica fluorescence microscope (Leica Microsystems Srl, Milan, Italy).

Statistical Analysis

All data in the text and figures are provided as means ± SEM. The results were analyzed by a one-way ANOVA and Tukey's test (SPSS 11.0 for Windows; SPSS Inc., Chicago, IL). $P < 0.05$ was considered significant.

RESULTS

The increase of Crocidolite-Induced LDH Leakage and Lipid Peroxidation (as 8-Isoprostane Detection) Is Prevented by Coincubation with Antioxidants

On the basis of previously published results (8, 13) and of the relative toxicity exerted by crocidolite, we incubated HMM cells with 25 μg/cm^2 and HMCs with 10 μg/cm^2 crocidolite for 24 hours as the most effective dose and time conditions. These concentrations of asbestos did not exert significant toxic effects (< 20% cell deaths) after a 24-hour incubation. HMCs were more sensitive than HMM cells to the crocidolite toxic effects.

After a 24-hour incubation with crocidolite, HMCs (Figure 1A and 2A) and HMM cells (Figure 1B and 2B) showed increased release of LDH (Figure 1) and augmented production of 8-isoprostane (Figure 2), used respectively as indexes of cytotoxicity and oxidative stress. These crocidolite effects were significantly

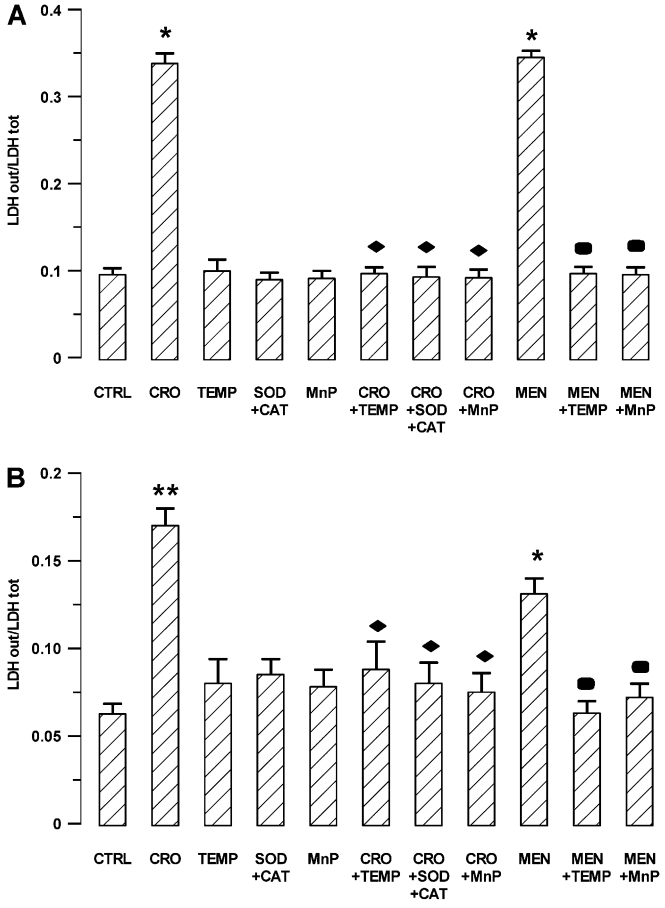

Figure 1. Effect of crocidolite and antioxidants on lactate dehydrogenase (LDH) leakage in human mesothelial cells (HMCs) (*A*) and in human malignant mesothelioma (HMM) cells (*B*). The cells were incubated for 24 hours in the absence (CTRL) or presence of the following compounds, alone or in different combinations: crocidolite fibers (CRO, 10 μg/cm^2 in HMCs or 25 μg/cm^2 in HMM cells), Tempol (TEMP, 200 μM), Mn-porphyrin (MnP, 20 μM), superoxide dismutase (SOD, 100 U/ml)/catalase (CAT, 1,000 U/ml), and menadione (MEN, 10 μM). Cells were then washed, detached, and checked for LDH activity in HMCs (*A*) and HMM cells (*B*). (*A*) Data are presented as means ± SEM ($n = 6$). Vs. CTRL: *$P < 0.001$. Vs. CRO: *diamond*, $P < 0.001$. Vs. MEN: *square*, $P < 0.001$. (*B*) Data are presented as means ± SEM ($n = 9$; i.e., three for each HMM cell line). Vs. CTRL: *$P < 0.001$; **$P < 0.0001$. Vs. CRO: *diamond*, $P < 0.001$. Vs. MEN: *square*, $P < 0.005$.

reversed in HMCs and HMM cells when they were incubated with the antioxidant agents Tempol (Sigma Chemical Co., St. Louis, MO), Mn-porphyrin (MnP), or the association superoxide dismutase/catalase (SOD/CAT) (Figures 1 and 2). A 24-hour incubation of the cells with menadione, used as a positive control of oxidative stress, induced LDH leakage and lipoperoxidation, which were prevented by the coincubation with antioxidants (Figures 1 and 2).

The Crocidolite-Induced Inhibition of RhoA Is Prevented by Antioxidants

GTP-bound RhoA can be used as an index of RhoA prenylation and activation (18). In HMCs (Figure 3A) and HMM cells (Figure 3B), a 24-hour incubation with crocidolite fibers markedly lowered the level of GTP-bound RhoA (Figures 3A and 3B), as we had previousy demonstrated in HMM cells (8). This effect was significantly prevented when crocidolite-treated cells were incubated with antioxidants, which *per se* did not modify the amount of GTP-bound RhoA (Figures 3A and 3B). Crocidolite lowered also the activity of ROCK in HMCs (Figure 4A) and HMM cells (Figure 4B), and again the antioxidants reversed this effect (Figures 4A and 4B). The incubation with menadione significantly inhibited GTP-bound RhoA and ROCK activities, which were prevented by Tempol coincubation (Figures 3 and 4).

The Crocidolite-Induced Inhibition of RhoA Prenylation Is Prevented by Antioxidants

A confirmation of RhoA inhibition and its reversion with antioxidants was assessed by investigating the RhoA prenylation. After crocidolite exposure, the RhoA prenylated form was sup-

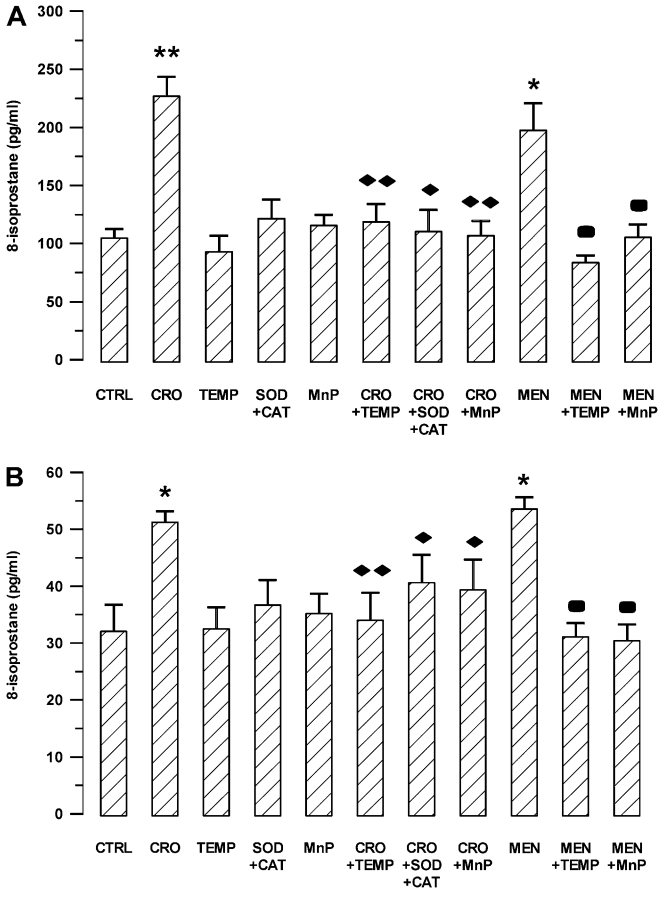

Figure 2. Effect of crocidolite and antioxidants on the production of 8-isoprostane in HMCs (*A*) and in HMM cells (*B*). The cells were incubated for 24 hours in the absence (CTRL) or presence of the following compounds, alone or in different combinations: crocidolite fibers (CRO, 10 μg/cm^2 in HMCs or 25 μg/cm^2 in HMM cells), Tempol (TEMP, 200 μM), Mn-porphyrin (MnP, 20 μM), superoxide dismutase (SOD, 100 U/ml)/catalase (CAT, 1,000 U/ml), menadione (MEN, 10 μM). Cells were then washed, detached and checked for 8-isoprostane production in HMCs (*A*) and HMM cells (*B*). (*A*) Data are presented as means ± SEM ($n = 6$). Versus CTRL: *$P < 0.001$; **$P < 0.0001$. Vs. CRO: *diamond*, $P < 0.001$; *double diamond*, $P < 0.0001$. Vs. MEN: *square*, $P < 0.002$. (*B*) Data are presented as means + SEM ($n = 9$; i.e., three for each HMM cell line). Vs. CTRL: *$P < 0.005$; **$P < 0.0001$. Vs. CRO: *diamond*, $P < 0.01$; *double diamond*, $P < 0.001$. Vs. MEN: *square*, $P < 0.05$.

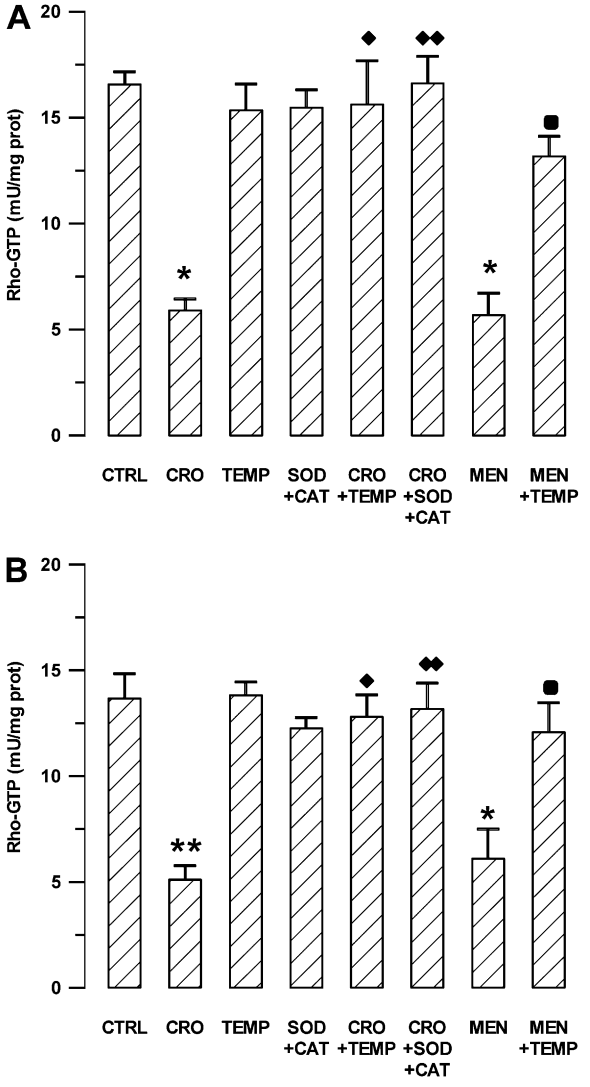

Figure 3. Effect of crocidolite and antioxidants on RhoA-GTP binding in HMCs (*A*) and in HMM cells (*B*). The cells were cultured for 24 hours in the absence (CTRL) or presence of the following compounds, alone or in different combinations: crocidolite fibers (CRO, 10 μg/cm^2 in HMCs or 25 μg/cm^2 in HMM cells), Tempol (TEMP, 200 μM), superoxide dismutase (SOD, 100 U/ml)/catalase (CAT, 1,000 U/ml), menadione (MEN, 10 μM). Subsequently, cells were lysed and checked for RhoA-GTP binding in HMCs (*A*) and HMM cells (*B*), as described under Materials and Methods. (*A*) Data are presented as means ± SEM ($n = 6$). Vs. CTRL: * $P < 0.001$. Vs. CRO: *diamond*, $P < 0.01$; *double diamond*, $P < 0.001$. Vs. MEN: *square*, $P < 0.05$. (*B*) Data are presented as means ± SEM ($n = 9$; i.e., three for each HMM cell line). Vs. CTRL: *$P < 0.05$; **$P < 0.001$. Vs. CRO: *diamond*, $P < 0.05$; *double diamond*, $P < 0.01$. Vs. MEN: *square*, $P < 0.05$.

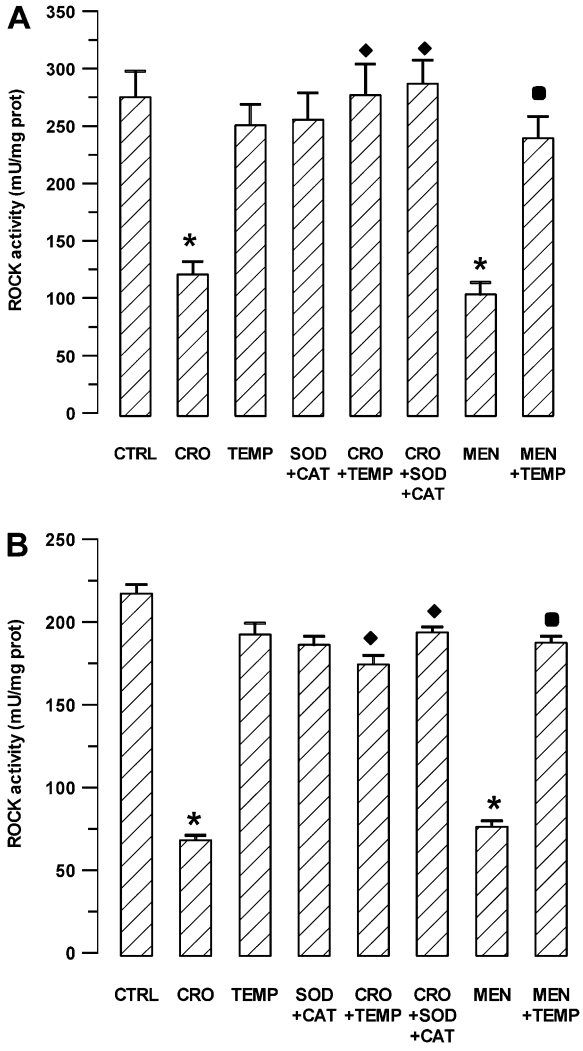

Figure 4. Effect of crocidolite and antioxidants on ROCK activity in HMCs (*A*) and in HMM cells (*B*). The cells were cultured for 24 hours in the absence (CTRL) or presence of the following compounds, alone or in different combinations: crocidolite fibers (CRO, 10 μg/cm^2 in HMCs or 25 μg/cm^2 in HMM cells), Tempol (TEMP, 200 μM), superoxide dismutase (SOD, 100 U/ml)/catalase (CAT, 1,000 U/ml), menadione (MEN, 10 μM). Subsequently, cells were lysed and checked for ROCK activity in HMCs (*A*) and HMM cells (*B*), as described under MATERIALS AND METHODS. (*A*) Data are presented as means ± SEM ($n = 6$). Vs. CTRL: *$P < 0.001$. Vs. CRO: *diamond*, $P < 0.01$. Vs. MEN: *square*, $P < 0.02$. (*B*) Data are presented as means ± SEM ($n = 9$; i.e., three for each HMM cell line). Vs. CTRL: *$P < 0.001$. Vs. CRO: *diamond*, $P < 0.01$. Vs. MEN: *square*, $P < 0.01$.

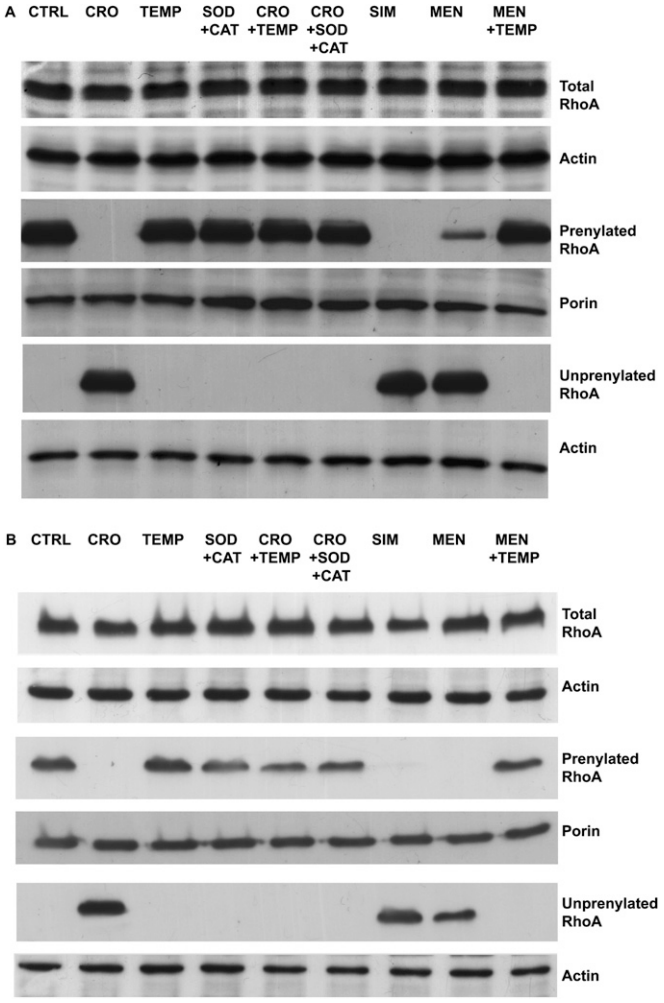

Figure 5. Effect of crocidolite and antioxidants on RhoA prenylation. The cells were treated as indicated in Figures 4 and 5 and the samples processed for TX-114 phase-partitioning. Simvastatin (SIM, 10 μM) was used as a known inhibitor of RhoA prenylation. Proteins from whole extracts, detergent-rich phase and aqueous fraction were analyzed for RhoA content by Western blotting in HMCs (*A*) and HMM cells (*B*). Actin and porin expression was evaluated as loading control. Blots are representative of a set of three independent experiments for HMCs and HMM cells, giving similar results.

pressed, while the unprenylated RhoA increased when compared with the control in HMCs (Figure 5A) and HMM cells (Figure 5B). Tempol and SOD/CAT completely reversed these effects (Figures 5A and 5B), without changing the expression of total RhoA (Figures 5A and 5B). Simvastatin was used as a known inhibitor of RhoA prenylation via HMGCR inhibition. The incubation with menadione significantly inhibited the RhoA prenylation, an effect prevented by the coincubation with Tempol (Figures 5A and 5B).

The Crocidolite-Induced Inhibition of HMGCR Activity Is Prevented by Antioxidants

To investigate the mechanism by which crocidolite-evoked ROS production inhibits the RhoA/ROCK pathway, we checked the activity of HMGCR, the enzyme responsible for the synthesis of isoprenoid molecules and essential for RhoA prenylation. After a 24-hour incubation with crocidolite, HMCs (Figure 6A) and HMM cells (Figure 6B) showed a significant decrease of HMGCR activity, whereas this event was completely prevented in the presence of antioxidants (Figures 6A and 6B). The incubation with menadione significantly inhibited the HMGCR activity, an effect prevented in the presence of Tempol (Figures 6A and 6B). Simvastatin, used as a known inhibitor of HMGCR activity, significantly inhibited HMGCR activity (Figures 6A and 6B).

The RhoA Inhibition Involves another Asbestos-Induced End Point, the Actin Organization in the Cytoskeleton

One of the most important end points downstream of RhoA activation concerns changes at the level of the cytoskeleton, in particular the modifications of actin organization. In the same experimental conditions shown above, we evaluated the actin alterations in fluorescently labeled cells. In HMM cells, crocid-

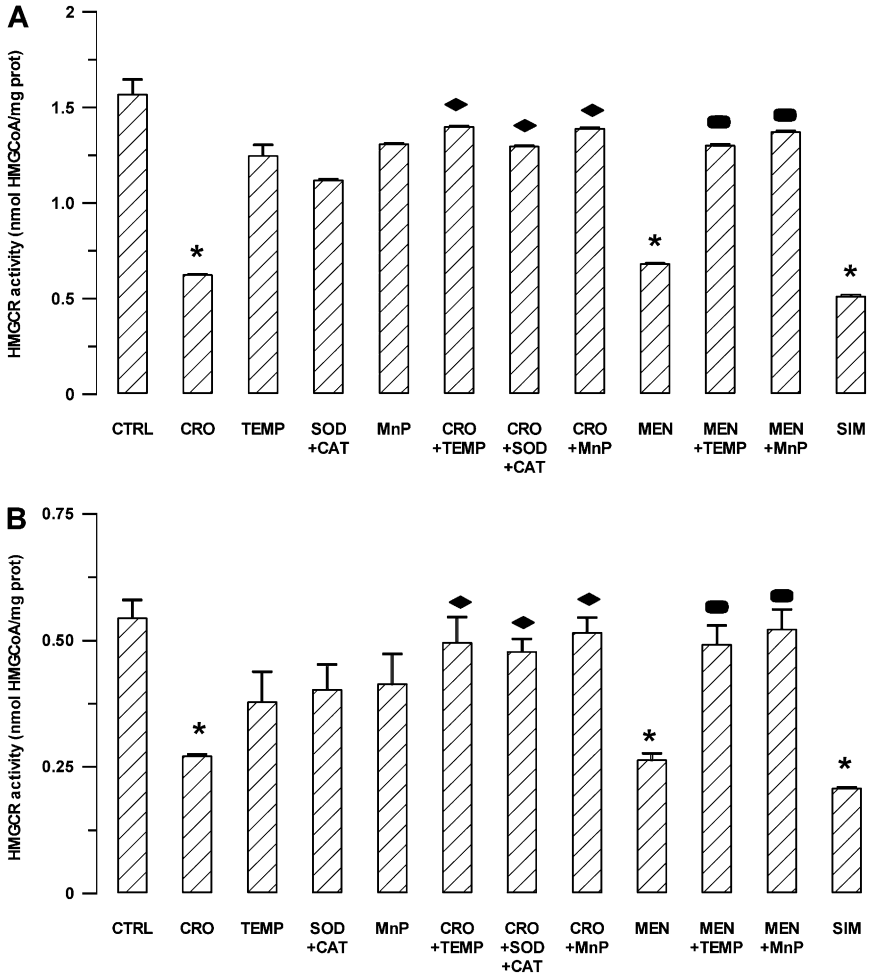

Figure 6. Effect of crocidolite and antioxidants on HMGCR activity in HMCs (*A*) and in HMM cells (*B*). The cells were cultured for 24 hours in the absence (CTRL) or presence of the following compounds, alone or in different combinations: crocidolite fibers (CRO, 10 μg/cm^2 in HMCs or 25 μg/cm^2 in HMM cells), Tempol (TEMP, 200 μM), Mn-porphyrin (MnP, 10 μM), superoxide dismutase (SOD, 100 U/ml)/ catalase (CAT, 1,000 U/ml), menadione (MEN, 10 μM), simvastatin (SIM, 10 μM). Subsequently, cells were lysed and checked for HMGCR activity, as described under MATERIALS AND METHODS. (*A*) Data are presented as means ± SEM ($n = 6$). Vs. CTRL: *$P <$ 0.0001. Vs. CRO: *diamond*, $P < 0.0001$. Vs. MEN: *square*, $P < 0.0001$. (*B*) Data are presented as means ± SEM ($n = 6$; i.e., two for each HMM cell line). Vs. CTRL: *$P < 0.0001$. Vs. CRO: *diamond*, $P <$ 0.0001. Vs. MEN: *square*, $P < 0.0001$.

olite as well as menadione and simvastatin modified the actin morphology, and this event was reversed by the antioxidant MnP (Figure 7). We obtained analogous data on HMCs (data not shown). These results suggest that the crocidolite-induced RhoA inhibition involves not only the effect on HMGCR but also on other RhoA-dependent events.

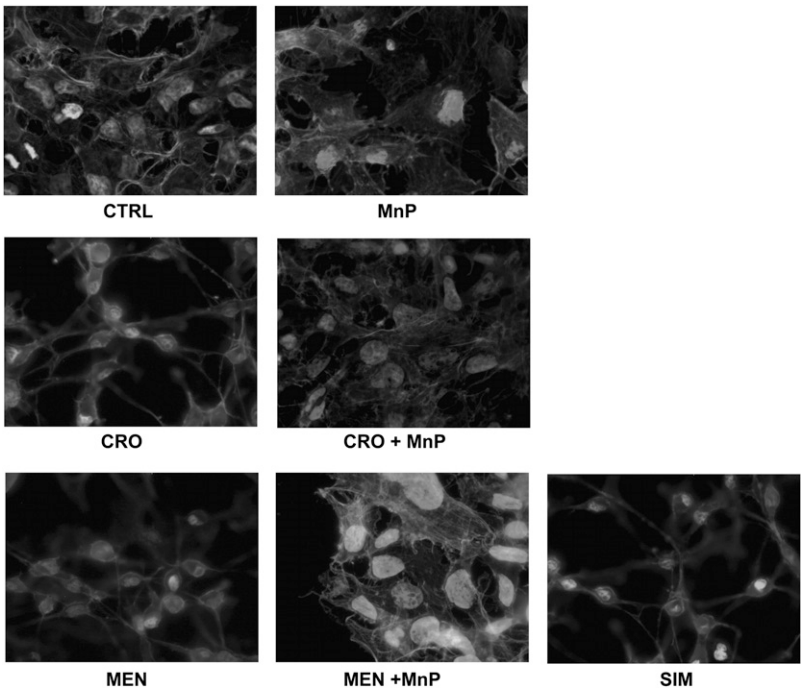

Figure 7. Effect of crocidolite and antioxidants on the actin organization in HMM cells. The cells were cultured for 24 hours in the absence (CTRL) or presence of the following compounds, alone or in different combinations: crocidolite fibers (CRO, 25 μg/cm^2), Mn-porphyrin (MnP, 10 μM), menadione (MEN, 10 μM), simvastatin (SIM, 10 μM). Subsequently, the cells were treated to detect the actin organization, as described under MATERIALS AND METHODS. Data are representative of a set of three independent experiments, giving similar results.

DISCUSSION

Chronic lung diseases, such as chronic obstructive pulmonary disease, asbestosis, silicosis, and cancer, are some of the major disorders induced by enhanced generation of ROS/reactive nitrogen species, which is strongly involved in the development of lung cancer (19). Asbestos induces cytotoxicity and apoptosis in HMCs (20, 21), as well as in other lung cell types (3, 4), and in such an effect the generation of superoxide and hydroxyl radicals has been deeply implicated. Some evidence suggests that cytotoxicity and mutagenesis induced by asbestos fibers are prevented by antioxidants and radical scavengers (22, 23), such as in lung epithelial (24, 25) and mesothelial cells (26). For this reason, we investigated whether ROS generation is involved in RhoA impairment in primary HMCs and HMM cell lines exposed to crocidolite.

The coincubation with the antioxidants Tempol, MnP, and SOD/CAT reversed the onset of crocidolite-induced cytotoxicity and oxidative stress, respectively, investigated as LDH release and 8-isoprostane production, a sensitive index of membrane lipid peroxidation. How ROS generation leads to cytotoxicity and genotoxicity of asbestos and other carcinogenic agents is matter of investigation, although high ROS levels and persistent oxidative stress have been recognized as characteristic features of carcinoma cells *in vivo* and *in vitro* (27).

In previous research, we observed that the incubation of HMM cells with crocidolite is associated with the nuclear translocation of the redox-sensitive factor NF-κB and that this activation involves a crocidolite-mediated RhoA inhibition (8). Our results suggested that the inhibition was at the level of HMGCR, the rate-limiting step in isoprenoid molecules synthesis, or at an upstream step, but until now the site and mechanism of the inhibition was not clear. The data of the present work show for the first time that crocidolite-induced RhoA/ROCK inhibition in HMCs and HMM cells is completely reversed by the coincubation with antioxidants. This complete reversal is detectable in terms of RhoA-GTP binding, ROCK activation, and RhoA prenylation, which is necessary to RhoA-GTP binding.

In our experiments, we caused the ROS increase by using menadione, a compound that exerts an oxidative stress by generating superoxide anion through its redox cycling and by forming a conjugate with glutathione (28): menadione significantly increased the oxidative stress, as evidenced by the increase of LDH release and 8-isoprostane production, and these effects were completely reversed by coincubation with Tempol or MnP. Similarly to crocidolite, menadione strongly inhibited RhoA-GTP binding, ROCK activation, and RhoA prenylation, and all these events were prevented by coincubation with Tempol. This suggests that an oxidative stress different from crocidolite exposure can induce the same pattern of response in HMCs and HMM cells.

The biosynthesis of isoprenoid compounds occurs via HMGCR, the rate-controlling enzyme in the sterol and nonsterol isoprenoid biosynthesis pathway, which is strictly regulated by different mechanisms, including, in bacteria and rats, the redox cell balance (9–11, 29). Our data provide further evidence for a similar regulation of HMGCR in humans. In fact, crocidolite exposure strongly inhibited HMGCR activity, and this effect was probably strictly related to the asbestos-induced production of cellular ROS. Indeed, we have demonstrated that the presence of antioxidants completely restored the enzyme activity.

Moreover, we have demonstrated that, in our experimental conditions, incubation with crocidolite fibers induces modifications of actin organization, a RhoA-dependent mechanism, and that this effect was reversed by the coincubation with antioxidants. This suggests that crocidolite incubation may affect other RhoA-dependent events besides HMGCR activity.

In conclusion, we propose that crocidolite-elicited ROS prevent RhoA prenylation by blocking the HMGCR activity and the subsequent geranylgeranyl pyrophosphate formation, necessary to the prenylation process, via the oxidation of the enzyme. The mechanism of such inactivation needs to be investigated. In this way asbestos, inhibiting RhoA, induces NF-κB activation and stimulates the synthesis of NO (8).

Although HMCs and HMM cells gave similar results in response to asbestos, the cytotoxic effect and the oxidative stress occurred in HMM cells at a higher concentration. In our opinion, this is in line with the evidence that, once generated, the HMM cells are more resistant than HMCs to the toxic effect exerted by crocidolite fibers. This is not the only effect of asbestos, and the molecular basis of crocidolite-associated toxicity and carcinogenesis is under investigation, but we have provided further insight into the complex series of events leading to asbestos-induced cytotoxicity, assuming that the crocidolite-mediated ROS production is considered an important signaling pathway in the transformation of normal mesothelial cells into a malignant mesothelioma.

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