High Levels of Catalase and Glutathione Peroxidase Activity Dampen H_2O_2 Signaling in Human Alveolar Macrophages

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Results are presented which support the hypothesis that adequate steady-state levels of hydrogen peroxide (H_2O_2) are required to overcome the effects of high catalase and glutathione peroxidase (GPx) expression for p38 mitogen-activated protein (MAP) kinase activation and tumor necrosis factor (TNF)-α gene expression in human alveolar macrophages stimulated with asbestos. We found significant differences in the types and amounts of reactive oxygen species generated in human blood monocytes compared with human alveolar macrophages. This difference in reactive oxygen species production is related, in part, to the differences in antioxidant enzyme expression and activity. Most importantly, catalase and GPx activities were significantly increased in alveolar macrophages compared with blood monocytes. Asbestos activated the p38 MAP kinase and induced TNF-α gene expression only in blood monocytes. Increasing the steady-state levels of H_2O_2 by using polyethylene glycol superoxide dismutase, an antioxidant that crosses the cell membrane, or aminotriazole, an irreversible inhibitor of catalase, allowed the p38 MAP kinase to be activated in alveolar macrophages. In addition, asbestos-stimulated macrophages cultured with polyethylene glycol superoxide dismutase had a significant increase in gene expression mediated by the TNF-α promoter. These results demonstrate that high catalase and GPx activity in human alveolar macrophages limits the effectiveness of H_2O_2 to act as a mediator of inflammatory gene expression.

Macrophages have an important role in initiating inflammatory responses by secreting proinflammatory cytokines. One important characteristic of the pathogenesis of asbestosis and other chronic lung diseases is an inflammatory response generated by a mixture of mature and immature macrophages. This is thought to result from the recruitment of monocytes to sites of disease. Some of these cells spontaneously release cytokines, which accentuate the inflammatory response. The significance of these observations has been illustrated in animal studies showing that inhibition of cytokines, such as tumor necrosis factor (TNF)-α, prevented the development of pulmonary fibrosis (1–3). These disorders are also associated with the generation of reactive oxygen species (ROS), which are involved in the inflammatory response by regulating signaling and can cause tissue injury when produced in high levels (4–6). The relative contribution of mature and immature macrophages to cytokine release and generation of ROS is unknown.

ROS are oxygen-containing molecules, such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), and hydroxyl radical ($HO^{\bullet}$), that have a greater chemical activity than molecular oxygen. ROS are generated in many inflammatory conditions in the lung and have been associated with cell injury and apoptosis (7, 8). All macrophages express a functional NADPH oxidase that is responsible for the reduction of oxygen to $O_2^{\bullet-}$ in the extracellular space. H_2O_2 generation results from the dismutation of $O_2^{\bullet-}$, which occurs at a rapid rate ($k = 10^5$–10^6 M^{-1} s^{-1}) nonenzymatically. Superoxide dismutase (SOD) increases the dismutation reaction by 10^4-fold (9–11). Studies have shown that asbestos directly activates the NADPH oxidase (12, 13), and asbestos has been shown to stimulate the generation of $O_2^{\bullet-}$ and H_2O_2 from alveolar macrophages cultured *in vitro* (14). The presence of oxidized proteins in lavage fluid obtained from patients with asbestosis provides evidence to support that ROS generation by these cells is relevant *in vivo* (15).

Studies have suggested that inflammation and cytotoxicity occur when an imbalance exists between ROS production and antioxidant enzyme activity (16, 17). Although excessive amounts of ROS can lead to inflammation and cytotoxicity, small alterations in ROS generation and antioxidant enzyme expression can result in activation of various signaling pathways resulting in mitogen-activated protein (MAP) kinase activation (6, 18–23) and cytokine gene expression (24–28). The MAP kinases are a family of second messengers that are essential for transferring signals from the cell surface to the nucleus. Studies indicate that MAP kinases are activated by asbestos (29, 30) and ROS (6, 18–23), and multiple studies have demonstrated the role of MAP kinases in cytokine gene expression (31–35). In addition, we have shown that the p38 MAP kinase is essential in activating basal transcription factors that are involved in regulating cytokine gene expression in macrophages (34–36).

In this study, we examined ROS generation in human alveolar macrophages and blood monocytes using asbestos as a model of ROS-associated disease. We also evaluated the role played by macrophages in regulating the activation of the p38 MAP kinase and cytokine gene expression. We hypothesized that immature macrophages, like blood monocytes, had more effective H_2O_2 levels both constitutively and with asbestos stimulation than alveolar macrophages. We found that the oxidant profile and the expression and activity of antioxidant enzymes in these cells depended on their level of differentiation. Most importantly, catalase and glutathione peroxidase (GPx) activities were significantly increased in alveolar macrophages compared with blood monocytes. Asbestos activated the p38 MAP kinase and induced TNF-α gene expression only in blood monocytes. Differentiated blood monocytes, like alveolar macrophages, stimulated with asbestos had an inactive p38 MAP kinase, and alveolar macrophages did not produce TNF-α. Increasing the steady-state levels of H_2O_2 by using polyethylene glycol (PEG)-superoxide

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Abbreviations: aminotriazole, ATZ; 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide, DEPMPO; dihydroethidium, DHE; 5,5-dimethyl-1-pyrroline *N*-oxide, DMPO; enzyme-linked immunosorbent assay, ELISA; electron spin resonance, ESR; glutathione peroxidase, GPx; Hanks' buffered saline solution, HBSS; horseradish peroxidase, HRP; lipopolysaccharide, LPS; mitogen-activated protein, MAP; nitroblue tetrazolium, NBT; superoxide anion, $O_2^{\bullet-}$; hydroxyl radical, $OH^{\bullet}$; phosphate-buffered saline, PBS; polyethylene glycol SOD, PEG-SOD; protein tyrosine phosphatase, PTP; reactive oxygen species, ROS; superoxide dismutase, SOD; tumor necrosis factor-α, TNF-α.

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dismutase (SOD), an antioxidant that crosses the cell membrane, or aminotriazole (ATZ), an irreversible inhibitor of catalase, allowed the p38 MAP kinase to be activated in alveolar macrophages. In addition, asbestos-stimulated macrophages cultured with PEG-SOD had a significant increase in gene expression mediated by the TNF-α promoter. These data suggest that the activation of the p38 MAP kinase and TNF-α gene expression in alveolar macrophages exposed to asbestos requires adequate steady-state levels of intracellular H_2O_2 to overcome the high level of catalase and GPx expression.

Materials and Methods

Cells

Alveolar macrophages and blood monocytes were obtained by bronchoalveolar lavage and by phlebotomy, respectively, from normal volunteers, and the Human Subjects Review Board of the University of Iowa College of Medicine approved this protocol. Normal volunteers had to meet the following criteria: (*i*) age between 18 and 45 yr; (*ii*) no history of cardiopulmonary disease or other chronic disease; (*iii*) no prescription or nonprescription medication except oral contraceptives; (*iv*) no recent or current evidence of infection; and (*v*) lifetime nonsmoker. The volunteers underwent phlebotomy and/or fiberoptic bronchoscopy with bronchoalveolar lavage after receiving intramuscular atropine, 0.6 mg, and local anesthesia. Each subsegment of the lung was lavaged with five 20-ml aliquots of normal saline, and the first aliquot in each was discarded. The percentage of alveolar macrophages was determined by Wright-Giemsa stain and varied from 90–98%. Blood monocytes were isolated by Ficoll-gradient centrifugation. Monocytes that were differentiated *in vitro* were cultured in RPMI with 10% human type AB serum for 7–10 d. RAW 264.7 cells were obtained from American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco's modified Eagle medium supplemented with gentamicin and 10% fetal calf serum.

Plasmids and Transfections

The pTRE-luc and pTET-ATF plasmids were obtained from Clontech. The TNF-luc (a generous gift from Dr. Dmitry V. Kuprash, Russian Academy of Sciences, Moscow, Russia) and the pcDNA-Cu,Zn-SOD plasmids have been previously described (37, 38). Transfections were performed utilizing the Effectene transfection reagent (Qiagen, Valencia, CA), according to the manufacturer's instructions. Twenty-four hours after transfection the cells were stimulated with crocidolite asbestos (NAIMA Fiber Repository) 5 μg/cm^2. Crocidolite asbestos [$Na_2(Fe^{3+})_2(Fe^{2+}{}_3Si_8O_{22}(OH)_2$] was used because it is known to cause progression of asbestosis compared with other fibers due to its estimated half-life on the order of decades (39). Luciferase activity, which was normalized to total protein, was measured after 6 h, which was determined to be the time of maximal activity.

Western Blot Analysis

Whole cell lysates were prepared by harvesting the cells after stimulating in the presence or absence of crocidolite asbestos for 15 min, and resuspending in lysis buffer (1% NP-40, 0.15 M NaCl, 0.05 M Tris [pH 7.4], 100 μg/ml phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 0.4 M Na_3VO_4, 10 mM NaF, and 10 mM sodium pyrophosphate). Samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and gels were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ), as previously described (34). The p-p38 monoclonal (Sigma, St. Louis, MO, and Biosource, Camarillo, CA), p38 MAP kinase rabbit polyclonal, Cu,Zn-SOD sheep polyclonal (Calbiochem, La Jolla, CA), human Mn-SOD rabbit (Upstate Biotechnology, Waltham, MA), and catalase rabbit (Athens Research and Technology, Athens, GA) polyclonal antibodies were used at dilutions of 1:1,000. PEG-SOD and catalase proteins were used at 75 and 100 U/ml, respectively.

Dihydroethidium Assay

Cells were loaded with dihydroethidium (DHE) for 20 min in phosphate-buffered saline (PBS) and then washed. Crocidolite asbestos 5 μg/cm^2 was added for 15 min in PBS, and then the cells were washed again. Fluorescent cells were counted in five fields and expressed as a percentage of total cells seen by light microscopy in each field.

Electron Spin Resonance Spectroscopy

Cells were placed in chelated PBS (pH 7.4). The spin traps, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DEPMPO), or 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), were added to the culture at a final concentration of 25–50 mM before stimulating the cells with crocidolite 5 μg/cm^2 for 15 min. The samples were then transferred to a flat cell, and spectra were collected for determination of $O_2^{\bullet-}$ and HO• generation. ESR spectra were recorded using a Bruker EMX electron spin resonance (ESR) spectrometer with the following settings: receiver gain, 1×10^6; modulation amplitude, 1.0 G; modulation frequency, 100.0 kHz; sweep width 80.0 G; microwave power, 40.1 mW; and frequency of 9.775 GHz. Spectra were the result of eight signal-averaged scans collected over 15 min. The analysis of the ESR spectra were based on the hyperfine splitting values characteristic of each spin adduct (40–42).

SOD and Catalase Activity Gel Assays

SOD and catalase activity were measured by separating samples on a native 12% or 8% polyacrylamide gel, respectively. For SOD, the gel was stained by incubation with 2.43 mM nitroblue tetrazolium (NBT), 28 μM riboflavin, and 28 mM TEMED for 20 min in the dark. For catalase, the gel was stained by incubation with 2% ferric chloride and 2% potassium ferricyanide.

Catalase and Glutathione Peroxidase Activity Assays

Catalase activity was determined using whole cell homogenates by measuring the exponential decay of 10 mM H_2O_2 ($\Delta\varepsilon_{240} = 39.4\ M^{-1} \cdot cm^{-1}$) in 50 mM potassium phosphate buffer, pH 7.0 monitored at 240 nm and the units expressed as *k* U/mg protein as previously described (43, 44). Total GPx activity was determined using 15 mM cumene hydroperoxide as substrate. Absorbance was read at 340 nm for 2.5 min. One unit of activity is defined as the amount of protein that oxidizes 1 μM of NADPH per min and is expressed as milli units per mg protein.

SOD Activity Assay

SOD activity of whole cell homogenates prepared on ice in 50 mM potassium phosphate buffer (pH = 7.8, with 1.34 mM diethylenetriaminepentaacetic acid) was determined using an indirect competitive inhibition assay (45). This assay is based on the competition between SOD and an indicator molecule NBT for superoxide production from xanthine and xanthine oxidase. One unit of activity was defined as that amount of protein required to inhibit NBT reduction by 50% of maximum for each cell type tested. Incubation for at least 45 min with 5 mM sodium cyanide was used to inhibit Cu,Zn-SOD activity to measure Mn-SOD activity. Cu,Zn-SOD activity was determined by subtracting Mn-SOD activity from total SOD activity.

Extracellular Measurement of H_2O_2

Measurement of H_2O_2 release from blood monocytes and alveolar macrophages was performed as previously described (46). This method takes advantage of the fact that H_2O_2 reacts with horseradish peroxidase (HRP)-forming compound I, which in turn reacts with *p*-hydroxyphenyl acetic acid (pHPA) forming a stable fluorescent dimer, $[pHPA]_2$. Cells were cultured in the presence or absence of crocidolite asbestos 5 μg/cm^2 in phenol red–free Hanks' buffered saline solution (HBSS; 1.0 ml) supplemented with glucose (6.5 mM), HEPES (1 mM), sodium bicarbonate (6 mM), pHPA (1.6 mM), and HRP (95 μg/ml). The amount of H_2O_2 released into the medium was followed spectrofluorometrically every 20 min over a period of 80 min at excitation and emission wavelengths of 323 and 400 nm, respectively. The fluorescent intensity of each sample was corrected for changes in pH and compared with standard concentrations of H_2O_2 determined by absorbance at 240 nm.

TNF-α Gene Expression

Blood monocytes and alveolar macrophages were cultured for 24 h in the presence or absence of crocidolite 5 μg/cm^2 or lipopolysaccharide (LPS) 1 μg/ml, which was used as a positive control. The supernatants

of the cells were harvested after 24 h, and TNF-α was measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN).

Statistical Analysis

All densitometry, enzyme activity, ELISAs, and luciferase measurements were normalized to total protein and are shown as means with the standard error. Statistical comparisons were performed using an unpaired, one-tailed *t* test or a two-way ANOVA with the probability of $P < 0.05$ considered to be significant.

Results

Alveolar Macrophages Generate More ROS than Blood Monocytes

Alveolar macrophages from normal individuals have a different phenotype compared with newly recruited monocyte-like cells in the lungs of patients with chronic lung disease. Therefore, we first determined the extent of ROS generation, including the levels of $O_2^{\bullet -}$ and HO•, in blood monocytes and alveolar macrophages. Initially, we evaluated ROS production in a nonspecific manner by loading the cells with DHE, which measures the production of $O_2^{\bullet -}$ as well as other oxidants. Crocidolite asbestos was added for 15 min in chelated PBS to avoid any iron contamination (47). White field and fluorescent photomicrographs were subsequently obtained (Figure 1A). More than 70% of control alveolar macrophages were fluorescent, and this remained > 70% in cells stimulated with crocidolite. In contrast, < 1% of blood monocytes were fluorescent in both the control state and after stimulation with crocidolite. The summation of the quantitative data from three different experiments is shown (Figure 1B). These studies demonstrate that alveolar macrophages spontaneously generate more ROS than do blood monocytes, and crocidolite asbestos does not trigger a detectable increase in ROS generation in either type of cell.

To evaluate the specific ROS generated in monocytes and macrophages, we used ESR spin-trapping to detect $O_2^{\bullet -}$ and HO• generation. To detect $O_2^{\bullet -}$ by ESR, we cultured the cells in chelated PBS to avoid any potential iron contamination. The spin trap, DEPMPO, was added to the culture before stimulating the cells with crocidolite. In some studies, PEG-SOD was used at a concentration of 75 U/ml, and was added to the culture before stimulation with crocidolite. We found that blood monocytes generated small levels of $O_2^{\bullet -}$ adducts of DEPMPO spontaneously, and the level of $O_2^{\bullet -}$ adducts detected was reduced when the cells were stimulated with crocidolite (Figure 1C). Alveolar macrophages generated significantly more $O_2^{\bullet -}$ adducts of DEPMPO spontaneously, but like the blood monocytes, stimulation with crocidolite slightly reduced the level of $O_2^{\bullet -}$ adducts detected, although it remained significantly greater than in the blood monocytes (Figure 1C). PEG-SOD abrogated the $O_2^{\bullet -}$-generated DEPMPO adducts in alveolar macrophages to a level comparable with asbestos-stimulated blood monocytes. These studies demonstrate that alveolar macrophages generate significantly more $O_2^{\bullet -}$ than blood monocytes both in the control state and when stimulated with crocidolite asbestos. The fact that

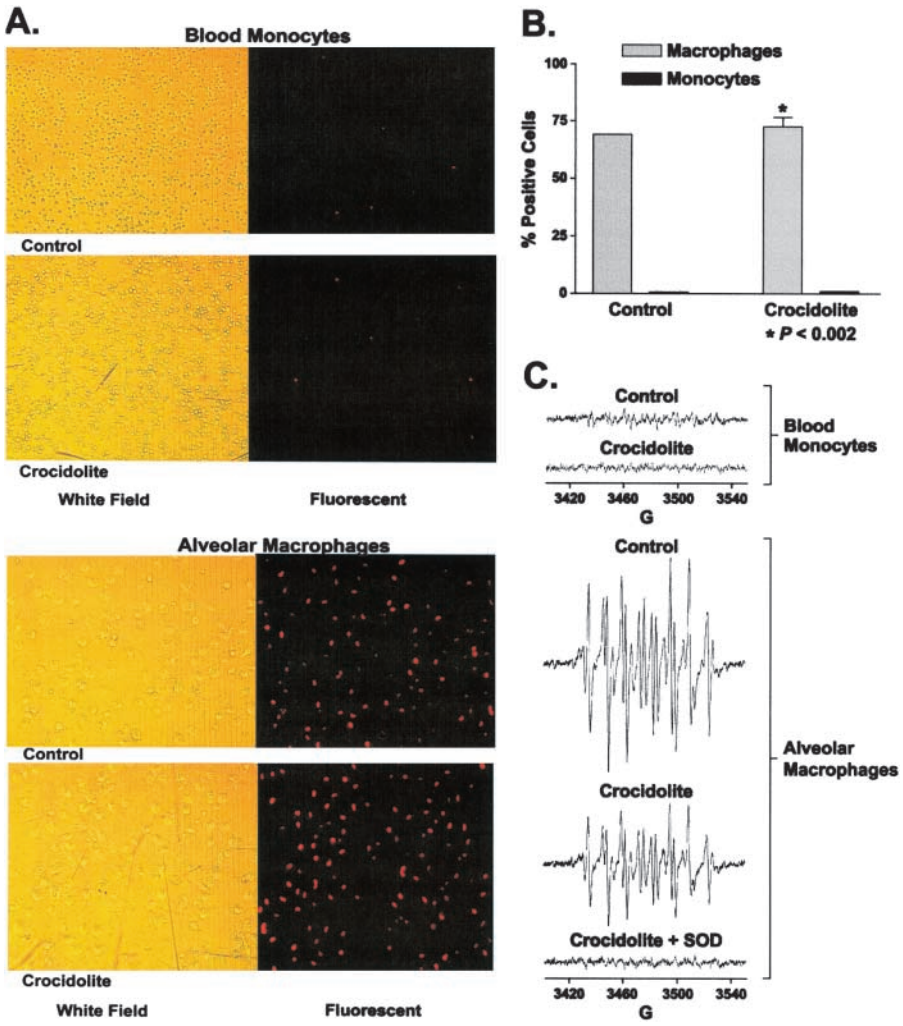

Figure 1. Alveolar macrophages generate more ROS than blood monocytes. (*A*) Cells were loaded with DHE in chelated PBS and then washed. Crocidolite was added in chelated PBS and then the cells were washed again. Representative photomicrographs of alveolar macrophages and blood monocytes are shown. (*B*) Graphical representation of the quantitative data from three separate DHE experiments. Fluorescent cells were counted in five separate fields and are expressed as a percentage of total cells in each field. Statistical comparisons of the asbestos-stimulated alveolar macrophages and blood monocytes were performed using an unpaired, one tailed *t* test. (*C*) Cells were cultured in chelated PBS, and the spin trap, DEPMPO, was added to the culture before stimulating the cells with crocidolite. When used, PEG-SOD was added to the culture prior to stimulation with crocidolite. Representative ESR spectra of blood monocytes and alveolar macrophages from five different experiments are shown. All ESR spectra are presented with the same ordinate scale. The spectra are a composite of species, DEPMPO/$O_2^{\bullet -}$ (a^N = 13.0 G, a^P = 50.4 G, a^H = 9.3 G; ~ 90%) and DEPMPO/HO• (a^N = 13.6 G, a^P = 48.1 G, a^H = 12.6 G; ~ 10%), consistent with a previous detailed analysis (41, 42).

PEG-SOD abrogates the ESR spectra confirms the generation of $O_2^{\cdot-}$ by alveolar macrophages in our experiments.

To detect HO•, we again used ESR with cells cultured in chelated-PBS. The spin trap, DMPO, was added before stimulation with crocidolite and/or the addition of PEG-SOD. Neither in the control state nor with asbestos stimulation did blood monocytes generate HO• adducts (Figure 2A). Blood monocytes differentiated over 10 d produced HO• adducts of DMPO both in the control state and with asbestos stimulation, although there was not a significant difference in DMPO/HO• adducts generated between these two groups (Figure 2B). In contrast, alveolar macrophages generated significant HO• adducts of DMPO in the control state, and this was augmented when the cells were stimulated with crocidolite (Figure 2C). When we cultured alveolar macrophages with PEG-SOD before stimulation with asbestos, we found that DMPO/HO• generation was abolished (Figure 2C). In addition, we performed a kinetic analysis of DMPO/HO• generation at 20-min intervals up to 80 min. The same overall pattern of HO• generation in the cells was consistent with the data at 15 min, although the amplitude slightly decreased with time in alveolar macrophages. However, alveolar macrophages generated HO• adducts of DMPO at 24 h (data not shown). We also found that deferoxamine decreased DMPO/HO• generation in asbestos-stimulated alveolar macrophages, but it did not affect $O_2^{\cdot-}$ production (data not shown). Taken together, these data demonstrate that alveolar macrophages produce more ROS, including $O_2^{\cdot-}$ and HO•, than their precursors, the blood monocytes, and that the process of differentiation into macrophages results in an increased ability to generate HO•. Furthermore, these data indicate that the spontaneous $O_2^{\cdot-}$ generation in alveolar macrophages primarily drives the Haber-Weiss reaction to produce HO•.

Crocidolite asbestos lowered $O_2^{\cdot-}$ generation in both blood monocytes and alveolar macrophages. In addition, asbestos increased the rate of HO• generation in macrophages. Because $O_2^{\cdot-}$ dismutates to H_2O_2 and HO• generation relies on H_2O_2 via the Fenton reaction, we next examined H_2O_2 production by these cells.

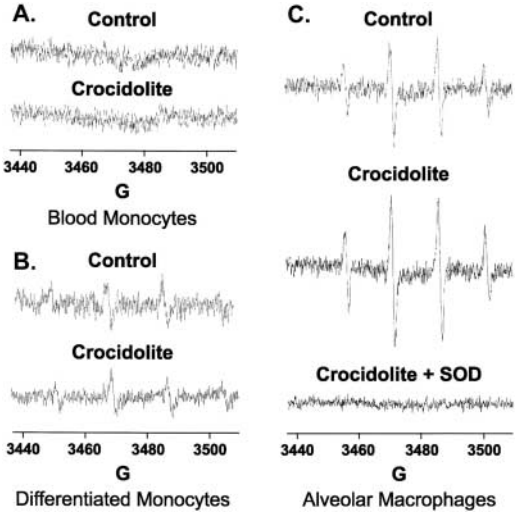

Figure 2. Alveolar macrophages generate HO• spontaneously and after stimulation with asbestos. Cells were cultured in chelated PBS, and the spin trap, DMPO, was added to the culture before stimulating the cells with crocidolite. When used, PEG-SOD was added to the culture before stimulation with crocidolite. Representative ESR spectra of (*A*) blood monocytes, (*B*) differentiated monocytes, and (*C*) alveolar macrophages from five different experiments are shown. All ESR spectra are presented with the same ordinate scale. The spectra are the result of DMPO/HO• ($a^N = a^H = 14.9$ G), consistent with previous report (40).

H_2O_2 Generation in Alveolar Macrophages is Significantly Enhanced with PEG-SOD

H_2O_2 is electrically neutral and considered to be an important second messenger (48, 49). Because H_2O_2 readily crosses the cell membrane, we measured extracellular H_2O_2, i.e., H_2O_2 released from the cells. Cells were cultured in the presence or absence of crocidolite asbestos for various amounts of time to determine the average amount of H_2O_2 released from the cell as a function of time. Blood monocytes spontaneously released H_2O_2 at a rate of 3.95 pmol/10^6 cells/min, and this approached statistical significance by increasing ($P < 0.079$) to 6.75 pmol/10^6 cells/min in asbestos-stimulated cells (Figure 3A and *inset*). Alveolar

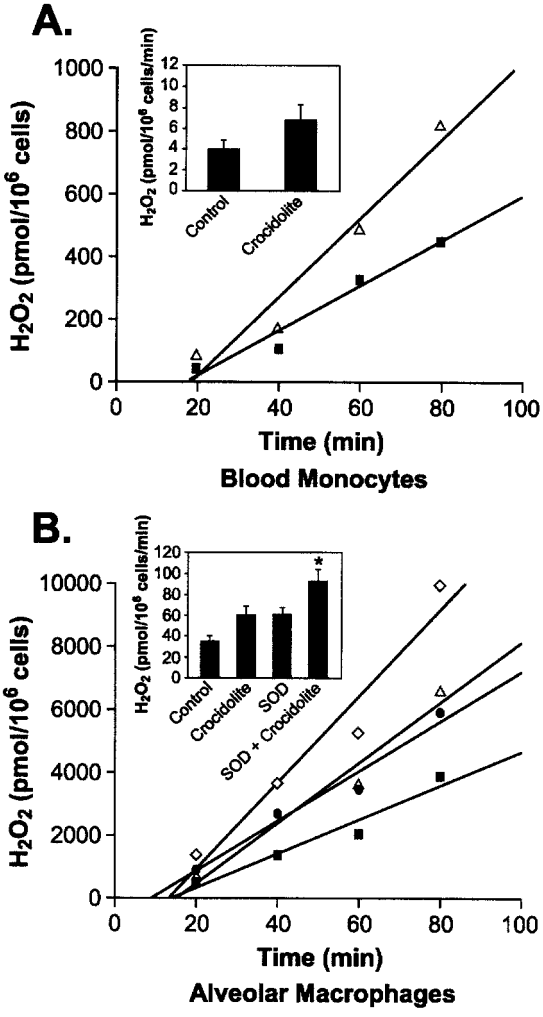

Figure 3. Alveolar macrophage H_2O_2 release is enhanced with PEG-SOD. Cells were cultured in the presence or absence of crocidolite asbestos in phenol red–free HBSS supplemented with glucose, HEPES, sodium bicarbonate, pHPA, and HRP. The amount of H_2O_2 released into the medium was followed spectrofluorometrically by measuring the formation of the fluorescent dimer $[pHPA]_2$ at excitation and emission wavelengths of 323 and 400 nm, respectively. Representative results of two experiments in (*A*) blood monocytes and (*B*) alveolar macrophages are shown. Statistical analysis was performed using two-way ANOVA comparing the slope (rate of generation) of the lines. These are shown in the *insets* of *A* and *B*. *Squares*, control; *triangles*, crocidolite; *circles*, SOD; *diamonds*, SOD + crocidolite.

macrophages spontaneously released H_2O_2 at a rate of 35.6 pmol/10^6 cells/min, and this was increased to 59.7 pmol/10^6 cells/min in cells stimulated with asbestos (Figure 3B). Thus, crocidolite asbestos increased the amount of extracellular H_2O_2 in both cells, but alveolar macrophages generated nine times the level generated in blood monocytes in both the control and the stimulated conditions.

Interestingly, in the presence of PEG-SOD, alveolar macrophages generated a rate of nearly 100 pmol of H_2O_2/10^6 cells/min (Figure 3B). This significantly enhanced rate of H_2O_2 generation ($P < 0.0001$) by alveolar macrophages was seen when stimulated with crocidolite asbestos (Figure 3B, *inset*). The reduction in $O_2^{\cdot-}$ after crocidolite stimulation in both blood monocytes and alveolar macrophages suggests that crocidolite enhances the dismutation of $O_2^{\cdot-}$ to H_2O_2. These steady-state levels of H_2O_2 are also dependent on the activity of peroxide-generating enzymes, such as SOD, and peroxide-removing enzymes, such as catalase and GPx.

SOD, Catalase, and GPx Activities Are Differentially Expressed in Blood Monocytes Compared with Alveolar Macrophages

Based on the differences in ROS generation between blood monocytes and alveolar macrophages and the necessity of $O_2^{\cdot-}$ for H_2O_2 and HO• production, we next evaluated the expression and activity of SOD in these cells. We first measured protein expression of Cu,Zn-SOD and Mn-SOD by Western blot analysis. Blood monocytes and alveolar macrophages were cultured in the presence or absence of crocidolite asbestos for 15 min to evaluate constitutive protein expression. High levels of Cu,Zn-SOD protein were expressed in blood monocytes compared with alveolar macrophages, whereas Mn-SOD protein was expressed at slightly greater levels in alveolar macrophages (Figure 4A). These data suggest that the difference in $O_2^{\cdot-}$ and HO• generation in these cells may be due, at least in part, to differences in the expression of the antioxidant enzyme SOD.

Because protein expression does not necessarily correlate with enzyme activity, we performed SOD activity gel assays to determine if enzyme activity mirrored protein expression. Cells were cultured in the presence or absence of crocidolite asbestos for 15 min. Native gel electrophoresis demonstrated that Cu,Zn-SOD activity was significantly increased in blood monocytes compared with alveolar macrophages, but crocidolite asbestos did not alter this activity. Mn-SOD activity was slightly increased after crocidolite exposure in blood monocytes (Figure 4B). In contrast, Cu,Zn-SOD activity was hardly detectable in alveolar macrophages, and Mn-SOD activity, although increased at baseline, was unchanged with crocidolite stimulation (Figure 4B). These results indicate that blood monocytes constitutively express active Cu,Zn-SOD that is not affected by asbestos stimulation and have inducible Mn-SOD activity. Alveolar macrophages, on the other hand, have significant Mn-SOD activity that is not altered by crocidolite asbestos stimulation.

Performing a solution assay for SOD activity corroborated our findings in the activity gel assays. Mn-SOD activity represented $\sim$ 35% of the total SOD activity in blood monocytes, and this activity increased slightly to $>$ 40% with crocidolite stimulation (Figure 4C). Mn-SOD activity represented $\sim$ 75% of the SOD activity in alveolar macrophages, and there was no change with exposure to crocidolite (Figure 4C). In contrast, Cu,Zn-SOD activity represented $>$ 65% of the total activity in blood monocytes and $\sim$ 25% in alveolar macrophages (Figure 4D). It is important to note that the total SOD activity (U/mg protein) in each cell was comparable (control: blood monocytes 138 ± 59 and alveolar macrophages 106 ± 10; crocidolite: blood monocytes 113 ± 30 and alveolar macrophages 138 ± 56), which

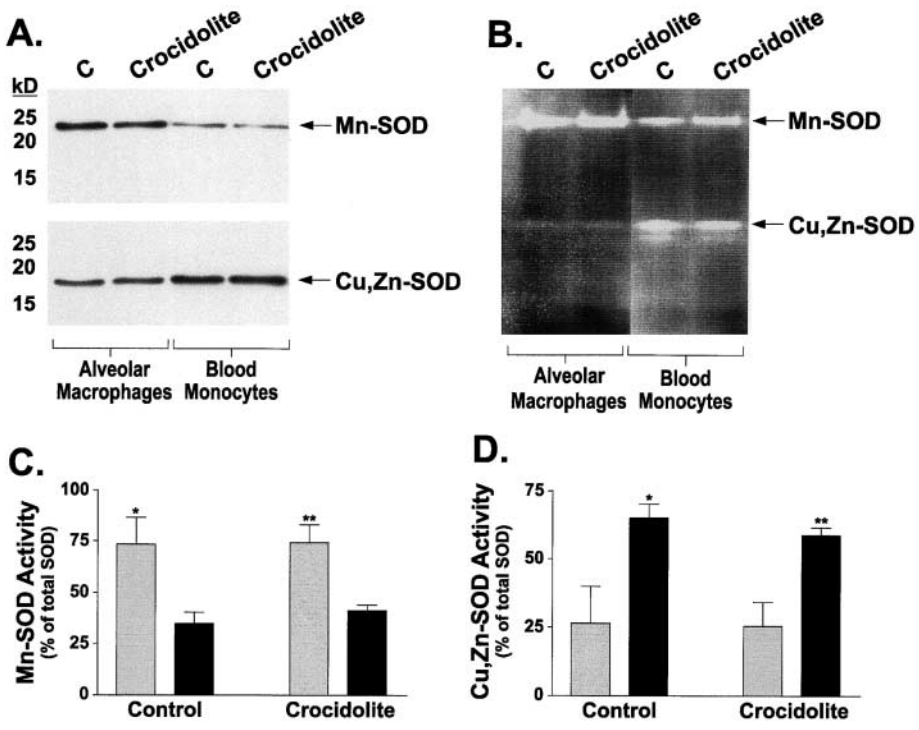

Figure 4. Cu,Zn-SOD is minimally active in alveolar macrophages. Cells were exposed to crocidolite. (*A*) Lysates were separated by SDS-PAGE. Western blot analysis p-p38 was performed with the Cu,Zn-SOD sheep polyclonal or Mn-SOD rabbit polyclonal antibodies. (*B*) Lysates were separated on a native polyacrylamide gel. The gel was then stained in the dark, and the achromatic bands corresponding to SOD appeared on a dark blue background in fluorescent light. (*C*) Whole cell homogenates were used in competition between SOD and NBT for superoxide production from xanthine 0.1 mM and xanthine oxidase 0.001 U/ml. The reaction is incubated at 37°C for 1 min and then measured spectrofluorometrically at 560 nm. One unit of activity was defined as that amount of protein required to inhibit NBT reduction by 50% of maximum for each cell type tested. Incubation for at least 45 min with 5 mM sodium cyanide was used to inhibit Cu,Zn-SOD activity to measure Mn-SOD activity. (*D*) Cu,Zn-SOD activity was determined by subtracting Mn-SOD activity from total SOD activity. Mn-SOD and Cu,Zn-SOD activities are expressed as percent of total SOD activity. Statistical comparisons in *C* and *D* are enzymes activities expressed as a percent of total SOD activity in control cells and asbestos-stimulated cells. These graphs are representative of three separate experiments. *Shaded bars*, macrophages; *solid bars*, monocytes. $*P < 0.028$, $**P < 0.012$.

suggests that there are differences in compartmentalization of SOD enzyme activity in monocytic cells depending on their state of differentiation.

We also evaluated the expression and activity of catalase and GPx in these cells and found a pattern opposite to that of SOD, especially Cu,Zn-SOD. We first measured protein expression of catalase by Western blot analysis. Cells were cultured in the presence or absence of crocidolite for 15 min. Catalase protein expression was slightly increased in alveolar macrophages compared with blood monocytes, and there was no change with crocidolite stimulation (Figure 5A). To determine catalase activity, we performed catalase activity gel assays and found that alveolar macrophages have significantly more activity than blood monocytes both constitutively and with asbestos stimulation (Figure 5B). Performing a solution assay for catalase activity corroborated our findings in the activity gel assays. Catalase activity in alveolar macrophages was significantly greater than in blood monocytes, but there was no significant change in activity with exposure to crocidolite asbestos in either cell (Figure 5C).

GPx activity assays were performed with a solution assay to determine total GPx activity in these cells. We found that total GPx activity in alveolar macrophages was significantly greater than in blood monocytes, but this activity decreased in alveolar macrophages and slightly increased in blood monocytes after stimulation with crocidolite asbestos (Figure 5D). In aggregate, these data show the differential expression of antioxidant enzymes in blood monocytes and alveolar macrophages, and this variation in expression may, in part, play a role in the ROS generation in these cells.

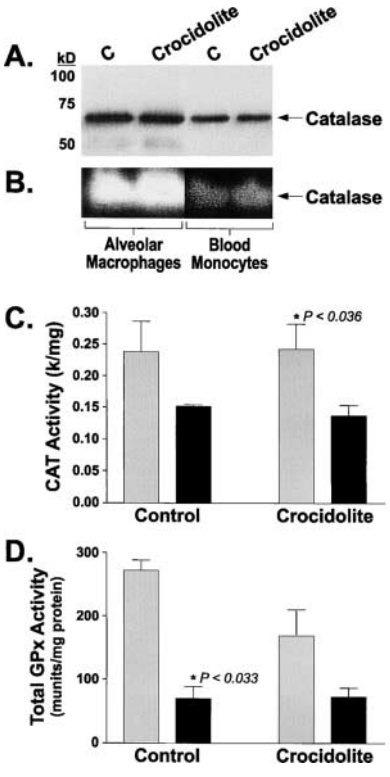

Figure 5. Alveolar macrophages express high catalase and GPx activity. Cells were exposed to crocidolite. (*A*) Lysates were separated by SDS-PAGE. Western blot analysis was performed with the catalase rabbit polyclonal antibody. (*B*) Lysates were separated on a native polyacrylamide gel. The gel was stained with 2% ferric chloride and 2% potassium ferricyanide. (*C*) Whole cell homogenates were used to measure the exponential decay of 10 mM H_2O_2 ($\Delta\varepsilon_{240} = 39.4 M^{-1}cm^{-1}$) in 50 mM potassium phosphate buffer, pH 7.0 monitored at 240 nm for 2 min. The catalase activity is expressed in *k* units/mg protein. (*D*) Whole cell homogenates were used to measure the degradation of 15 mM cumene hydroperoxide. Absorbance was read at 340 nm for 2.5 min. One unit of activity is defined as the amount of protein that oxidizes 1 μM of NADPH per min and is expressed as milli units per mg protein. Statistical comparisons in *C* were of asbestos-stimulated alveolar macrophages and blood monocytes and in *D* of control alveolar macrophages and blood monocytes. These graphs are representative of three separate experiments. *Shaded bars*, macrophages; *solid bars*, monocytes.

SOD Induces p38 MAP Kinase Activity in Alveolar Macrophages

Due to the disparity in ROS generation and antioxidant enzyme activity in blood monocytes compared with alveolar macrophages, we determined if there was any functional difference in other enzyme activity. We evaluated the activation of the p38 MAP kinase because it is redox-regulated, and our previous studies demonstrated that this kinase regulates transcription factors necessary for cytokine gene expression in macrophages (34–36). Blood monocytes, differentiated monocytes, and alveolar macrophages were cultured in the presence or absence of asbestos for 15 min, which was determined to be the time of maximal p38 activation. We found that crocidolite asbestos strongly activated the p38 MAP kinase in blood monocytes as measured by the presence of the phosphorylated form of the kinase (Figure 6A). In contrast, neither differentiated monocytes nor alveolar macrophages had an active p38 MAP kinase after asbestos stimulation. Western blot analysis for the p38 MAP kinase total protein shows equal loading of proteins (Figure 6A). The densitometry of three separate experiments in blood monocytes and

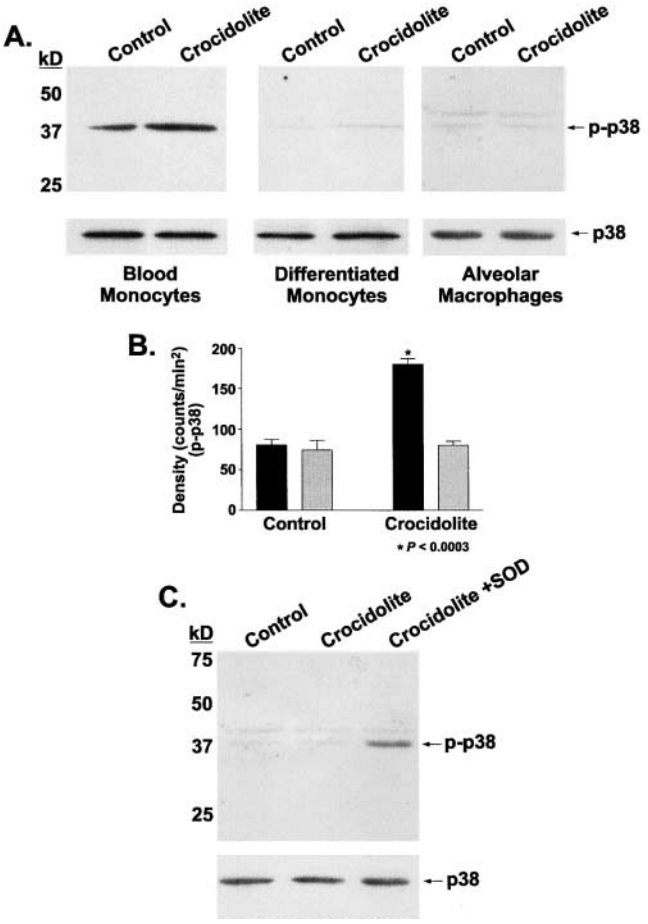

Figure 6. SOD induces p38 MAP kinase activation in alveolar macrophages. (*A*) Cells were exposed to crocidolite 5 μg/cm² for 15 min. (*B*) Densitometry of three separate Western blot analyses of blood monocytes (*solid bars*) and alveolar macrophages (*shaded bars*). Statistical comparisons of the asbestos-stimulated cells were performed using an unpaired, one-tailed *t* test. (*C*) Alveolar macrophages were cultured in the presence or absence of PEG-SOD 75 U/ml before being stimulated with crocidolite. All lysates were separated by SDS-PAGE. Western blot analysis was performed with the p-p38 monoclonal or the p38 rabbit polyclonal antibodies to determine activation and confirm equal loading of proteins, respectively.

alveolar macrophages is shown (Figure 6B). These data show the absence of p38 MAP kinase activation in mature monocytic cells stimulated with crocidolite asbestos, whereas their precursor cells, the blood monocytes, have an active p38 MAP kinase with asbestos stimulation.

We next determined if increasing the steady-state levels of H_2O_2 with PEG-SOD could induce p38 MAP kinase activity in alveolar macrophages stimulated with asbestos. Alveolar macrophages were cultured with PEG-SOD for 15 min before asbestos stimulation. Crocidolite asbestos did not activate the p38 MAP kinase in alveolar macrophages, but cells cultured with PEG-SOD recovered p38 MAP kinase activity with asbestos stimulation (Figure 6C). Taken together, these data show that SOD activity regulates activation of the p38 MAP kinase in monocytic cells stimulated with asbestos. In addition, these data suggest that the Cu,Zn-SOD can influence the steady-state levels of cytosolic H_2O_2, and thus, is important for p38 MAP kinase activation in monocytic cells stimulated with asbestos.

H_2O_2 Activates the p38 MAP Kinase

Due to the fact that alveolar macrophages have increased $O_2^{\cdot-}$ and HO· generation, decreased Cu,Zn-SOD activity, and increased catalase and GPx activity compared with blood monocytes, we asked if H_2O_2 was responsible for activating the p38 MAP kinase in monocytic cells. The premise for these experiments is that SOD catalyzes the dismutation of $O_2^{\cdot-}$ to H_2O_2, and may, therefore, influence the steady-state levels of H_2O_2 (50, 51). We first determined if catalase inhibited asbestos-induced p38 MAP kinase activation in blood monocytes. Blood monocytes were cultured in the presence or absence of catalase protein 100 U/ml for 15 min before asbestos stimulation. Crocidolite asbestos strongly activated the p38 MAP kinase in blood monocytes, as demonstrated by the presence of the phosphorylated form of the kinase, and catalase inhibited both the baseline and the activation by crocidolite to below control levels (Figure 7A).

Due to the increased activity of catalase in alveolar macrophages and to confirm that H_2O_2 was responsible for p38 activity after crocidolite asbestos stimulation, we used ATZ, an irreversible inhibitor of catalase, to increase intracellular levels of H_2O_2 in alveolar macrophages. Alveolar macrophages were cultured in the presence or absence of ATZ (20 mM) before stimulation with asbestos. Cells cultured in the presence of ATZ for 1 h had a slight increase in p38 MAP kinase activation, and this activation was augmented with crocidolite stimulation (Figure 7B). Longer incubations (3 h) with ATZ alone or with crocidolite stimulation resulted in a greater increase in p38 MAP kinase activation in alveolar macrophages, and stimulation with crocidolite did not significantly alter this activation (Figure 7B). Taken together, these data strongly support the hypothesis that adequate levels of intracellular H_2O_2 must be present for p38 MAP kinase activation in monocytic cells.

To ensure that ATZ was indeed increasing H_2O_2 generation in alveolar macrophages, we measured H_2O_2 levels in cells cultured in the presence or absence of ATZ (20 mM) with or without crocidolite asbestos stimulation. We found that the steady-state level of H_2O_2 was significantly enhanced in the presence of ATZ, and crocidolite did not significantly alter the level of H_2O_2 generated (Figure 7C). The data confirm that adequate steady-state levels of H_2O_2 are necessary for p38 MAP kinase activation in alveolar macrophages. In aggregate, these data also suggest that, although alveolar macrophages release excessive amounts of H_2O_2 compared with blood monocytes, their high levels of catalase and GPx activity limits the effectiveness of H_2O_2 as a second messenger to activate the p38 MAP kinase.

To further demonstrate that H_2O_2 mediates p38 MAP kinase activation and that the role of Cu,Zn-SOD is to increase the accumulation of cytoplasmic H_2O_2 to an adequate level, we performed an *in vivo* p38 MAP kinase assay in RAW 264.7 cells, a murine macrophage cell line that has many of the features of primary alveolar macrophages, such as ROS production (52, 53). In this assay, when ATF is phosphorylated by the p38 MAP kinase, the phosphorylated ATF can bind to the tetracycline-response element (TRE) in the luciferase reporter promoter to induce luciferase expression. RAW 264.7 cells were co-transfected with the TRE luciferase reporter plasmid, pTET-ATF, and either an

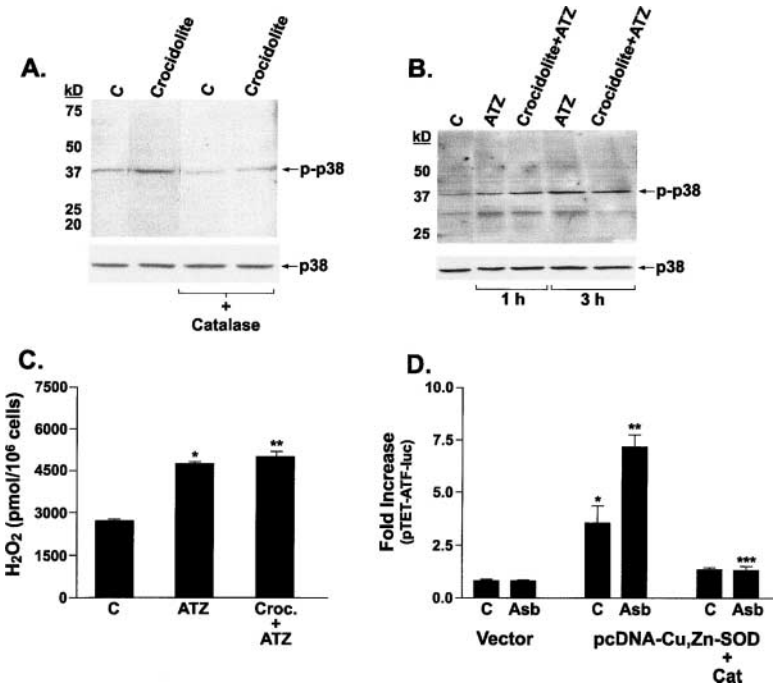

Figure 7. H_2O_2 activates the p38 MAP kinase. (*A*) Blood monocytes were cultured in the presence or absence of catalase 100 U/ml before being stimulated with crocidolite 5 μg/cm^2 for 15 min. (*B*) Alveolar macrophages were cultured in the presence or absence of ATZ 20 mM for either 1 h or 3 h before stimulation with crocidolite 5 μg/cm^2. For *A* and *B*, lysates were separated by SDS-PAGE. Western blot analysis was performed with the p-p38 monoclonal or the p38 rabbit polyclonal antibodies to determine activation and confirm equal loading of proteins, respectively. (*C*) Alveolar macrophages were cultured in the presence or absence of ATZ 20 mM with or without crocidolite asbestos for 1 h in phenol red–free HBSS supplemented with glucose, HEPES, sodium bicarbonate, pHPA, and HRP. The amount of H_2O_2 released into the medium was followed spectrofluorometrically by measuring the formation of the fluorescent dimer $[pHPA]_2$ at excitation and emission wavelengths of 323 and 400 nm, respectively. This graph is representative of three experiments. $^*P < 0.0001$, $^{**}P < 0.0001$. (*D*) RAW 267.4 cells were transiently co-transfected with pTRE-luc, pTET-ATF, and either an empty vector or pcDNA-Cu,Zn-SOD. After 24 h, the cells were cultured in the presence or absence of catalase 100 U/ml before being stimulated with crocidolite 5 μg/cm^2 for 6 h. Luciferase activity, which is normalized to protein, is expressed as fold increased from control. This graph is representative of three experiments. $^*P < 0.0450$, $^{**}P < 0.0045$, $^{***}P < 0.0060$.

empty vector or the pcDNA-Cu,Zn-SOD expression vector in the presence or absence of catalase (100 U/ml). Similar to the human alveolar macrophages, crocidolite asbestos did not activate the p38 MAP kinase in cells transfected with the empty vector. In contrast, cells expressing Cu,Zn-SOD had increased luciferase activity in control cells, and this was significantly increased with asbestos stimulation (Figure 7D). In cells expressing Cu,Zn-SOD in the presence of catalase, luciferase activity was inhibited to control levels (Figure 7D). In aggregate, these data strongly support the conclusion that asbestos-induced p38 MAP kinase activation in monocytic cells is dependent upon adequate steady-state levels of intracellular H_2O_2. These data also demonstrate that the increase in catalase and GPx activity is associated with the absence of p38 MAP kinase activation in alveolar macrophages stimulated with crocidolite asbestos.

SOD Induces TNF-α Gene Expression in Macrophages

To determine if the recovery of p38 activity had any physiologic relevance, we measured TNF-α production in blood monocytes and alveolar macrophages. Because the p38 MAP kinase was activated in blood monocytes stimulated with crocidolite asbestos, we cultured blood monocytes with different doses of crocidolite (0.5–15 μg/cm^2) for 24 h. We found that crocidolite asbestos induced significant TNF-α production at every concentration, but the most maximal response was at 5 μg/cm^2 (Figure 8A). In contrast, TNF-α release in asbestos-stimulated alveolar macrophages was no different than control (Figure 8B). LPS was included in experiments with alveolar macrophages as a positive control.

To evaluate if enhancing H_2O_2 generation by increasing SOD activity would recover the ability to express TNF-α, we transiently transfected RAW 264.7 cells with TNF-luc. After 24 h, the cells were cultured in the presence or absence of PEG-SOD for 15 min prior to stimulating with crocidolite asbestos for 6 h. We found that asbestos-stimulated cells treated with PEG-SOD had a significant increase—almost 12-fold—in gene expression mediated by the TNF-α promoter, as measured by luciferase activity (Figure 8C). In aggregate, these data indicate that adequate steady-state levels of H_2O_2, which can be obtained by over expression of SOD, are necessary for p38 MAP kinase activity and TNF-α gene expression in alveolar macrophages stimulated with asbestos.

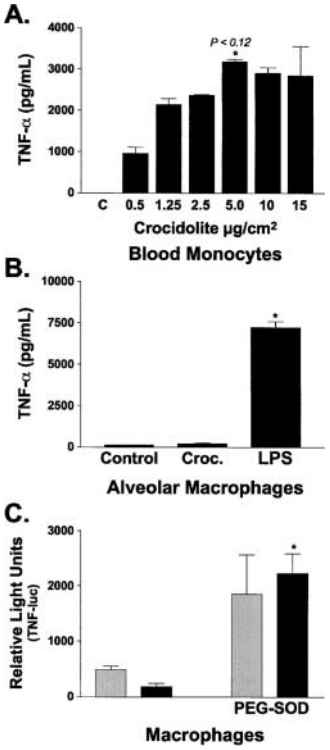

Figure 8. SOD induces TNF-α gene expression in macrophages. (*A*) Blood monocytes were cultured for 24 h with various doses of crocidolite. (*B*) Alveolar macrophages were cultured for 24 h with crocidolite 5 μg/cm^2 or LPS 1 μg/ml, as a positive control. $*P <$ 0.001. (*C*) RAW 267.4 cells were transfected with TNF-luc. After 24 h, cells were exposed to crocidolite for 6 h in the presence or absence of PEG-SOD 75 U/ml. Luciferase activity, normalized to protein, is expressed as raw relative light units. *Shaded bars*, control; *solid bars*, crocidolite. $*P <$ 0.016. In *A* and *B*, cell supernatants were harvested, and TNF-α protein was measured by ELISA. Each graph is representative of three experiments.

Discussion

In this study, we have demonstrated that p38 MAP kinase activation and TNF-α gene expression in human monocytic cells stimulated with asbestos is dependent upon adequate steady-state levels of H_2O_2. We also demonstrated that blood monocytes and alveolar macrophages have differential expression of ROS and that alveolar macrophages are relatively resistant to H_2O_2 secondary to their high level of catalase and GPx activity. A novel aspect of our study is that SOD, particularly Cu,Zn-SOD activity, can dramatically influence p38 MAP kinase activation and cytokine gene expression in monocytic cells stimulated with asbestos by its ability to influence steady-state levels of intracellular H_2O_2 and, thus, overcome the effects of catalase and GPx. Therefore, H_2O_2 appears necessary for p38 MAP kinase activation and TNF-α gene expression in monocytic cells stimulated with a relevant oxidant stimulus, such as crocidolite asbestos.

Asbestos can enhance the production of ROS in the lung by stimulating cells recruited to the area of insult, i.e., the site of asbestos deposition. In our study, we found a striking difference in the $O_2^{\bullet-}$, HO•, and H_2O_2 levels obtained in alveolar macrophages compared with blood monocytes. ROS have been shown to affect various signaling pathways, including the MAP kinase pathways (6, 18–23). These studies show that both $O_2^{\bullet-}$ and H_2O_2 act as mediators of MAP kinase activation. The direct mechanism by which $O_2^{\bullet-}$ activates MAP kinases appears to be via the classically described pathways (6, 18). Although both monocytes and macrophages generate $O_2^{\bullet-}$ via activation of the NADPH oxidase, we do not believe $O_2^{\bullet-}$ is responsible for p38 MAP kinase activation because it is not membrane permeable. It is highly unlikely that HO• is involved in signaling because it is so highly reactive and causes immediate oxidation of molecules that it encounters (54, 55). It is plausible, however, that HO• generation in asbestos-stimulated alveolar macrophages is inhibitory depending on the location where it is produced. That is, lipid peroxidation may occur if HO• is generated near the cell membrane, and this could result in the inhibition of upstream kinases that activate p38. Studies to evaluate this possibility are currently ongoing in our laboratory.

There is evidence that alveolar macrophages from patients with asbestosis function differently than macrophages from the lungs of normal volunteers (15, 56). The cells from patients with asbestosis and other chronic lung diseases have been shown to be younger or "monocyte-like" cells (56–60). In addition, studies have reported functional differences in cytokine release when normal alveolar macrophages are compared with blood monocytes using other stimuli or when compared with alveolar macrophages from patients with chronic lung disorders (61–65). These studies suggest that the "monocyte-like" cells in the lungs of patients with asbestosis are responsible for releasing cytokines. Our results support these previous observations due to the fact that only blood monocytes release TNF-α when stimulated with asbestos.

In contrast to $O_2^{\bullet-}$ and HO•, H_2O_2 has several characteristics of a second messenger. These characteristics include being generated rapidly after a stimulus, the ability to freely diffuse within the cell and across the cell membrane, reacting at specific sites, and being degraded enzymatically. More importantly, H_2O_2 is electrically neutral and has a significantly longer half-life than other ROS (66). We found that H_2O_2 mediated p38 MAP kinase

activation due to the fact that catalase inhibited p38 activity in monocytes, which have low catalase and GPx activity. To recover p38 activity in alveolar macrophages, which have high expression and activity of both catalase and GPx, we used PEG-SOD or ATZ to enhance H_2O_2 generation and/or overcome the enzymatic degradation of H_2O_2. One study has demonstrated that high catalase activity is associated with increased cell viability and resistance to H_2O_2 in macrophages (67). Taken together, our results show that adequate steady-state levels of H_2O_2 must be attained for p38 MAP kinase activation and TNF-α gene expression in monocytic cells stimulated with asbestos. These results also suggest that the levels of H_2O_2 required in alveolar macrophages for asbestos to activate p38 or express TNF-α are much greater than the levels required in blood monocytes due to their high level of catalase and GPx activity.

Although it is clear from our results that H_2O_2 is responsible for activation of the p38 MAP kinase in asbestos-stimulated monocytic cells, we did not specifically evaluate the mechanism by which the kinase is activated. One plausible mechanism could be through the inactivation of a protein-tyrosine or dual-specific phosphatase. These phosphatases contain highly conserved cysteine residues that are essential for catalytic activity, and studies indicate that micromolar concentrations of H_2O_2 can oxidize these essential cysteine residues to form a sulfenic acid (Cys-SOH) intermediate (68–71). Three such phosphatases, PTP2, PTP3, and HePTP, have been shown to directly dephosphorylate the tyrosine residue in the activation loop of p38 MAP kinase (72, 73). Furthermore, studies suggest that cysteine modifications are important regulators of signal transduction (74, 75). It will be of interest in future studies to identify the redox-sensitive phosphatase(s) involved in the oxidant-mediated activation of the p38 MAP kinase.

The contrast between the levels of ROS generated in alveolar macrophages and blood monocytes appears to be relevant to the activation of signaling pathways and gene expression; that is, the small alterations in ROS generation and antioxidant enzyme expression in blood monocytes results in p38 MAP kinase activation and TNF-α gene expression. The significant difference in ROS generation in these cells can be attributed, in part, to the dissimilarity in SOD expression and catalase and GPx expression. Although SOD gene expression has been shown to differ between monocytes and macrophages (76, 77), the functional relevance of this difference has not been explained. Previous studies show that total SOD activity increases in the lung after exposure to asbestos, but only Mn-SOD mRNA and protein levels change in response to asbestos or other ROS (17, 78). We find similar results in blood monocytes, but alveolar macrophages had minimal Cu,Zn-SOD activity and lacked inducible Mn-SOD expression or activity. Cu,Zn-SOD and Mn-SOD are also known to influence the generation of H_2O_2 (50, 51, 79, 80). To our knowledge, only one study has demonstrated that SOD influences steady-state levels of H_2O_2 that resulted in increased expression of matrix metalloproteinase-1 (20). Thus, the difference in the constitutive activity of Cu,Zn-SOD and the inducibility of Mn-SOD is the likely mechanism for the disparity in $O_2^{\bullet-}$ and HO• generation in these cells. The constitutive activity of Cu,Zn-SOD is of primary importance in monocytic cells due to the early time point (15 min) of p38 MAP kinase activation after asbestos stimulation. Our results indicate that H_2O_2 is participating in the activation of the p38 MAP kinase and TNF-α gene expression in alveolar macrophages and blood monocytes. These results also suggest that SOD, particularly Cu,Zn-SOD, has the unique role of regulating signal transduction in monocytes by controlling the cytosolic redox environment of the cell by increasing steady-state levels of H_2O_2.

Although asbestos has profound effects on epithelial cells and fibroblasts in the lung, the focus of this study was to determine the effects of ROS generation and antioxidant expression in regulating inflammatory genes in alveolar macrophages using a relevant oxidant stimulus, such as asbestos. More specifically, the current study demonstrates that the activation of the p38 MAP kinase and TNF-α gene expression by crocidolite asbestos in human monocytic cells is redox-sensitive. This is the first study to show that the imbalance in ROS production and antioxidant enzyme expression in human alveolar macrophages prevents the activation of signaling pathways important in initiating immune and/or inflammatory responses. This imbalance appears to result in inadequate generation of steady-state levels of H_2O_2 secondary to high levels of catalase and GPx activity.

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