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Original Contribution

GRP78, HSP72/73, AND CJUN STRESS PROTEIN LEVELS IN LUNG EPITHELIAL CELLS EXPOSED TO ASBESTOS, CADMIUM, OR H_2O_2

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Abstract—Occupational exposure to crocidolite asbestos is associated with the development of nonmalignant and malignant pulmonary disease. Considerable evidence indicates that the mechanisms of asbestos-induced toxicity involve the production of active oxygen species (AOS). Production of AOS in excess of cellular defenses creates an environment of oxidative stress and stimulates the expression of a number of different genes whose products may be involved in mediating responses from oxidant injury. To further investigate the mechanisms of asbestos-induced pathogenicity, we have examined by Western blot analyses the induction of the stress response proteins GRP78 and HSP72/73 in rat lung epithelial cells (RLE) exposed to crocidolite asbestos. In comparative studies, we also examined GRP78, HSP72/73, and cJun expression in RLE cells exposed to equitoxic concentrations of cadmium chloride ($CdCl_2$) and hydrogen peroxide (H_2O_2). Our results demonstrate that asbestos and H_2O_2 do not alter GRP78 or HSP72/73 protein levels in RLE cells, but do increase levels of cJun protein. Increases by asbestos and H_2O_2 were not accompanied by alterations in cellular glutathione levels in this cell type, but asbestos caused elevations in protein levels of manganese-containing superoxide dismutase (MnSOD), an indirect indicator of oxidant stress. In contrast, exposure of cells to $CdCl_2$ led to no changes in MnSOD protein levels, but increases in GRP78, HSP72/73, and cJun proteins as well as significant increases in oxidized and reduced thiol pools. Results suggest that environmental agents causing oxidative injury to lung epithelium elicit different patterns of stress responses.

Keywords—Asbestos, Epithelial cells, Stress response, $CdCl_2$, H_2O_2, GRP78, HSP72/73, cJun, Free radical

INTRODUCTION

Asbestos, a family of fibrous silicate minerals, is associated with the development of malignant and nonmalignant pulmonary disease.[1,2] Previous studies using in vitro cultures of pulmonary and pleural cells have shown that exposure of cells to asbestos can stimulate a number of dose-related responses including cell proliferation,[3] changes in normal cell differentiation,[4] and cell death.[5] Many of these asbestos-induced effects in vitro appear to be mediated by active oxygen species (AOS, i.e., superoxide anion, hydroxyl radical) catalyzed directly on the fiber surface or released from phagocytic and inflammatory cells.[6,7] AOS are potentially damaging to cells because they can interact with and modify a number of macromolecules including oxidative modification of DNA[8] and lipid peroxidation.[9] A role of AOS in asbestos-induced cell injury and disease is suggested in studies examining the effects of antioxidants on cell survival and development of pulmonary fibrosis.[6,7,10] For example, concomitant exposure of cultured pulmonary cells to antioxidants[10,11] or stable transfection of cells with a plasmid overexpressing MnSOD[12] can inhibit asbestos-induced cytotoxicity. Furthermore, in an animal model of asbestosis, administration of polyethylene glycol-conjugated catalase, a scavenger of AOS, during inhalation of asbestos ameliorates pulmonary toxicity, inflammation, and the development of disease.[13]

Excessive production of AOS, creating an environment of oxidative stress, can result in loss of cell function and cell death. As a consequence, mammalian cells have developed elaborate defense systems to scavenge excess AOS, repair oxidant-induced damage, and adapt to the changes in their environment.[9] The spectrum of genes induced during these responses depends both on the cell

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type and on the particular toxicant to which the cell is exposed. Included in the cellular response to AOS is induction of antioxidant enzymes,[9] and early-response genes,[14–17] e.g., c-*jun*, and synthesis of a group of highly conserved stress proteins, the glucose regulated proteins (GRPs),[18] and the heat-shock proteins (HSPs).[19]

An elaborate defense system of antioxidant enzymes and scavengers of AOS (glutathione, ceruloplasmin, etc.) occur in different compartments in the lung. Previous work in our laboratory has demonstrated that inhalation of asbestos causes increased mRNA levels of the antioxidant enzymes MnSOD, copper–zinc superoxide dismutase, and glutathione peroxidase (GPX) in rat lungs.[20] Furthermore, the activities of total superoxide dismutase, catalase, and GPX are increased in rat lung after inhalation of asbestos.[20,21] These studies indicate that inhalation of asbestos elicits an adaptive response against oxidant-induced stress in pulmonary cells.

The proto-oncogene, c-*jun*, is a member of a multigene family that is transiently expressed in response to a variety of stimuli and encodes subunits (Jun homodimers and Jun/Fos heterodimers) of the transcription factor AP-1.[22] c-*jun* and members of the c-*fos* gene family are immediate early-response genes involved in cell cycle control and are implicated in the responses of cells to oxidative stress.[22,23] Previous work in our laboratory has demonstrated that exposure of tracheal epithelial or pleural mesothelial cells in vitro to asbestos elicits increased expression of the early-response genes c-*jun*, c-*fos*,[17] and c-*myc*,[24] Asbestos-induced expression of c-*jun* and c-*fos* is also accompanied by increased AP-1 DNA binding activity[17] and increased transcription of AP-1–dependent genes.[25] Moreover, steady-state mRNA levels of c-*jun* are elevated in rat lung after exposure to crocidolite asbestos.[26]

GRP78[18] and HSP72/73[19,27,28] (constitutive and inducible forms of HSP70, respectively) are highly conserved proteins that function during normal cellular metabolism as molecular chaperones to mediate the correct folding, translocation, and processing of newly synthesized proteins. HSP72/73 is located in the cytoplasm where it binds nascent polypeptides, as they are synthesized preventing aggregation and misfolding during processing and translocation to various cellular compartments.[19,28] GRP78, located in the lumen of the endoplasmic reticulum (ER), serves a similar function during translocation and processing of membrane bound and secretory proteins in the ER.[18] GRP78 is also a calcium binding protein and may play a role in Ca^{2+} homeostasis.[18] During the stress response, GRP78 and HSP72/73 are thought to sequester denatured or damaged proteins within the cell, thus preventing their aggregation, assisting in the refolding of denatured proteins, or facilitating their degradation.[19,28] The expression of both proteins is increased at the transcriptional level by a variety of toxic agents that damage proteins or interfere with protein processing, i.e., proteotoxic agents.[28] Included in this group are a number of toxicants that mediate their effects through the production of AOS.[29–33]

In the work presented here, we examined expression of GRP78 and HSP72/73 stress proteins in rat lung epithelial cells (RLE) exposed to crocidolite asbestos. In comparative experiments, we also examined the effect of cadmium chloride ($CdCl_2$) and the oxidant, H_2O_2, on GRP78, HSP72/73, and cJun expression in RLE cells. The toxicity of $CdCl_2$, a suspect lung carcinogen, has been examined in other cell types in which it induces the expression of the c-*jun*[34] and *hsp*70.[29,35] Moreover, $CdCl_2$ has been implicated as an agent inducing oxidative injury as a mechanism of toxicity.[29] H_2O_2 is a product of the inflammatory response and has recently been implicated as a carcinogen in urinary epithelial cells.[36] In studies here, we also examined the levels of oxidized (GSSG) and reduced (GSH) glutathione and protein levels of MnSOD in RLE cells exposed to equitoxic concentrations of asbestos, $CdCl_2$, or H_2O_2 to relate changes in stress protein levels to patterns of oxidative stress.

MATERIALS AND METHODS

Asbestos fibers, $CdCl_2$, and H_2O_2

Reference samples of National Institute of Environmental Health (NIEHS) processed crocidolite asbestos were obtained from the Thermal Manufacturers Association Fiber Repository (Littleton, CO). The fiber characterization and size dimensions of this preparation have been reported previously.[13] Prior to addition to cell cultures, asbestos fibers were sterilized at 225°F for 12–15 h, suspended in Hanks Balanced Salt Solution (HBSS, GIBCO) at 1 mg/ml and triturated eight times through a 22-gauge needle. $CdCl_2$ (Sigma, St. Louis, MO) was dissolved in HBSS and filter sterilized before addition to cultures. H_2O_2 (Sigma) was diluted in calcium and magnesium-free phosphate-buffered saline (CMFPBS) and added directly to culture medium.

Cell culture, exposure to test agents, and cytotoxicity

RLE cells are a spontaneously immortalized rat type II alveolar epithelial cell line generously obtained from K. Driscoll[37] (The Proctor and Gamble Company, Cincinnati, OH). Cells were propagated in DMEM/F12 medium (GIBCO BRL) containing 10% newborn calf serum (NBS, GIBCO) and used between passages 31 to 41. Twenty-four hours prior to exposure to test agents, the

growth medium on confluent cultures was changed to fresh DMEM/F12 supplemented with 0.5% NBS. Asbestos fibers were added directly to medium at final concentrations of 1, 5, and 10 $\mu g/cm^2$ area of culture dish. Freshly prepared $CdCl_2$ and H_2O_2 were added to the culture medium at final concentrations of 1, 5, and 10 μM $CdCl_2$ and 100, 200, and 500 μM H_2O_2. For each experiment, duplicate dishes ($n = 2$) for untreated or treated cultures were assayed. The cytotoxicity of each agent in RLE cells was determined by trypan blue exclusion and cell counting.[11] All experiments were performed in duplicate. Briefly, at the indicated times, cells were harvested by trypsinization, an aliquot of the cell suspension was mixed with an equal volume of trypan blue (GIBCO BRL), and the number of viable or dead (blue cells taking up the dye) were counted using a hemocytometer. Viability was expressed as the percentage of living cells excluding dye divided by the total number of cells counted. All results were evaluated with one-way analysis of variance.

Western blot analysis

Cells were washed twice with cold CMFPBS, and proteins extracted in lysis buffer (20 mM Tris-HC1 pH7.4, 150 mM NaCl, 1% Nonidet P40 (Boehringer–Mannheim, Indianapolis, IN), 2 mM dithiothreitol (Sigma), 1 mM sodium orthovanadate (Sigma), 1 mM phenylmethylsulfonsyl fluoride (Sigma), 10 μg/ml leupeptin (Sigma), and 10 mg protein/ml aprotinin (Sigma).[38] Lysates were transferred to 1.5 ml Eppendorf tubes and incubated for 30 min on ice. Cell debris was pelleted at 4°C for 20 min at 14,000 rpm in a microcentrifuge. An aliquot of the supernatant was removed for determination of protein content using the Bio-Rad protein assay (BioRad, Hercules, CA). The remaining lysate was mixed with an equal volume of 2× sample buffer (5 mM EDTA, 4% sodium dodecyl sulfate, 40% B-mercaptoethanol, 20% glycerol, 0.2 M Tris-base pH6.8, and saturated bromphenol blue) and stored at −20°C until analysis. Equal amounts of each protein sample (15 μg) were electrophoresed in 12% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred to nitrocellulose using a semidry transfer apparatus (Ellard Instrumentation Ltd, Seattle, WA). Blots were blocked overnight at 4°C in CMFPBS plus 5% powdered milk. Following a 1 h incubation at RT° in CMFPBS/5% milk plus 0.05% Tween-20 (Sigma), blots were incubated at RT° with a specific primary antibody for 45 min, washed 3× with CMFPBS/0.05% Tween-20, and incubated with peroxidase-conjugated secondary antibody for 30 min at RT. Blots were washed 3× in CMFPBS/0.05% Tween-20, once with CMFPBS and protein bands visualized with the LumiGlo enhanced chemiluminescence detection system (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Rabbit anti-GRP78 was purchased from StressGen (Victoria, B.C.). Mouse anti-HSP72/73 (#sc-24) and mouse anti-cJun (#sc-822) were purchased from Santa Cruz (Santa Cruz, CA) and anti-human kidney MnSOD was generously provided by L. Oberly (University of Iowa, Iowa City, IA). Autoradiographs were quantitated by densitometric analysis using a Microtex scanner and HP Deskscan and Sigma Scan 3.0 analysis software. All experiments were performed in duplicate.

Glutathione assay

Cells were washed once with CMFPBS, scraped from the culture dish in 2 ml CMFPBS, and transferred to centrifuge tubes. An aliquot of each cell suspension was removed, lysed by freeze-thaw lysis, and analyzed for protein content as described above. The remaining cell suspensions were centrifuged at 1200 rpm to pellet the cells. Cell pellets were then lysed in 250 μl 25% TCA in phosphate-EDTA buffer (0.1 M $NaPO_4$/ 0.005 M EDTA pH 7.8). The lysates were transferred to Eppendorf microcentrifuge tubes, the volume adjusted to 1 ml with phosphate-EDTA buffer, and centrifuged at 14,000 rpm for 2 min. The supernatants were transferred to clean microcentrifuge tubes and stored at −80°C prior to analysis. Determination of reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were performed by the fluorometric method of Hissin and Hilf.[39] For determination of GSH, 100 μl of sample was mixed with 100 μl of freshly prepared *o*-phthalaldehyde (OPT, 1 mg/ml in methanol) (Sigma), the total volume adjusted to 2 ml with phosphate-EDTA buffer, and incubated for 15 min at RT. Fluorescence intensity was determined with excitation and emission wavelengths of 320 nm and 420 nm, respectively using a Hitachi F-2000 fluorescence spectrophotometer. A standard curve using reduced glutathione (Sigma) was generated for a range of concentrations. For determination of GSSG, 100 μl of sample was mixed with 40μl of 0.04 M *N*-ethylmaleimide (Sigma) for 30 min at RT, the total volume adjusted to 2 ml with 0.1 N NaOH, and subsequently incubated with 140 μl OPT for 15 min at RT. A standard curve was generated using oxidized glutathione (Sigma) at various concentrations.[40] Fluorescence intensity was determined as described above for GSH. A total of two experiments were performed to evaluate GSSG levels, and three experiments were performed for evaluation of GSH levels. All results were evaluated with one-way analysis of variance.

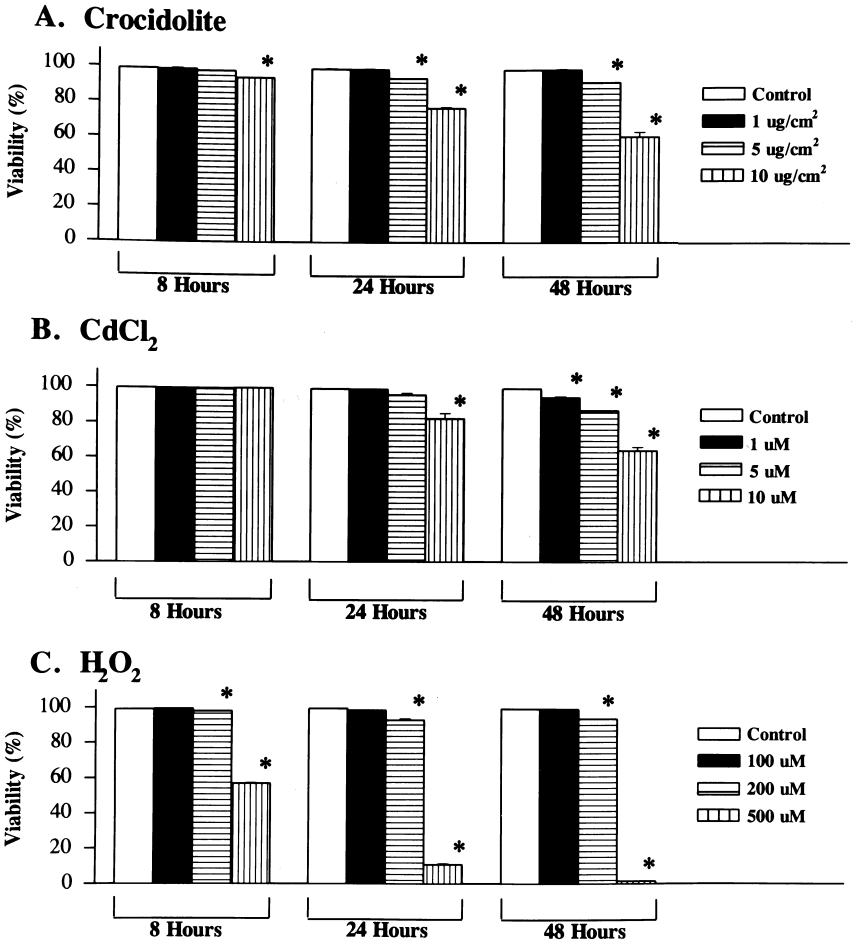

Fig. 1. Viability of RLE cells exposed to crocidolite asbestos, $CdCl_2$, or H_2O_2. RLE cells were grown to confluence and the culture medium switched to 0.5% serum 24 h prior to the addition of test agents. After 8, 24, and 48 h of exposure to crocidolite asbestos (1, 5, or 10 $\mu g/cm^2$), $CdCl_2$ (1, 5, or 10 μM), or H_2O_2 (100, 200, or 500 μM), cells were harvested by trypsinization and cell viability was assessed using trypan blue exclusion and cell counting. Results are expressed as the percentage of viable cells in comparison to total cells counted (mean ± SEM). $*p < .05$.

RESULTS

Cytotoxicity of crocidolite asbestos, $CdCl_2$, or H_2O_2 in RLE cells

To establish kinetic and dose parameters of cytotoxicity in cells exposed to crocidolite, $CdCl_2$, or H_2O_2, we examined, using the trypan blue exclusion technique, the viability of RLE cells at 8, 24, and 48 h following exposure to various concentrations of each agent (Fig. 1). The viability of RLE cells exposed to crocidolite asbestos is shown in Fig. 1A. At all time points examined, exposure to asbestos at 1 $\mu g/cm^2$ had no effect on cell viability. Slight, but statistically significant, decreases in viability were observed following exposure of cells to 5 $\mu g/cm^2$ asbestos for 24 and 48 h. At the highest concentration of asbestos examined (10 $\mu g/cm^2$), cell viability was significantly decreased at all time points.

In cells exposed to $CdCl_2$ for 8 h, we observed no significant changes in cell viability (Fig. 1B). At 24 h of exposure, 10 μM $CdCl_2$ caused a significant decrease in viability. A dose-dependent decrease in viability was observed after 48 h of exposure to $CdCl_2$ at all concentrations. No significant changes in viability were detected at any time point for RLE cells exposed to 100 μM H_2O_2. In contrast, exposure of cells to 200 or 500 μM H_2O_2 caused significant decreases in RLE cell viability at all time points examined (Fig. 1C). Because the levels of cytotoxicity observed in RLE cells exposed to 10 μM $CdCl_2$ or 200 μM H_2O_2 were comparable to levels of cytotoxicity in cells exposed to 10 $\mu g/cm^2$ or 5 $\mu g/cm^2$

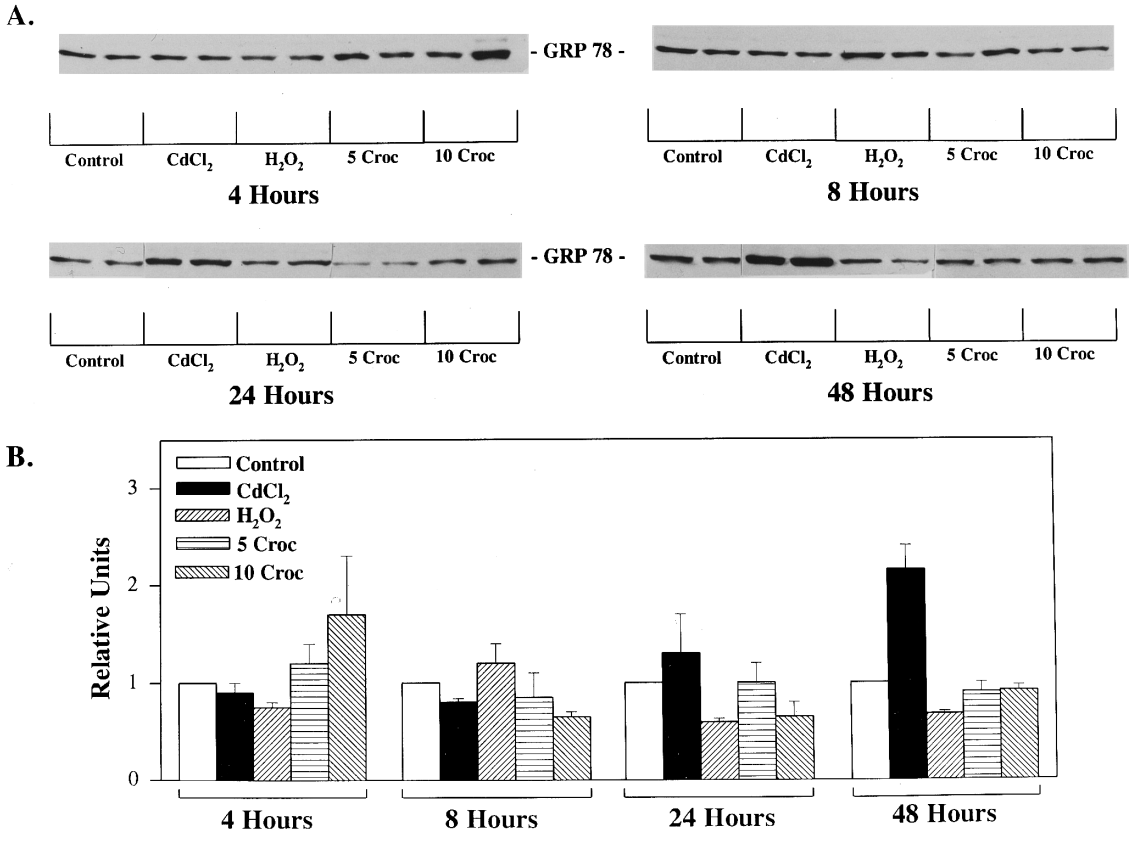

Fig. 2. Western blot analysis of GRP78 protein levels in RLE cells after exposure to crocidolite asbestos, $CdCl_2$, or H_2O_2. RLE cells were grown to confluence, and the culture medium switched to 0.5% serum 24 h prior to the addition of test agents. After 4, 8, 24, and 48 h of exposure to asbestos (5 and 10 μg/cm^2), $CdCl_2$ (10 μM), or H_2O_2 (200 μM), total protein was isolated, and 15 μg of each sample was electrophoresed on 12% SDS-polyacrylamide gels and transferred to nitrocellulose. Blots were incubated with a primary antibody specific for GRP78 as described in Materials and Methods. (A) Representative Western blot analysis of GRP78 protein levels in RLE cells exposed to various agents. (B) Densitometric analysis of GRP78 Western blot. Results are expressed as mean ± SEM.

asbestos, respectively (Fig. 1A, B, and C), we chose these approximately equitoxic doses of each agent to be used in studies below.

Protein levels of GRP78 and HSP72/73 in RLE cells after exposure to crocidolite asbestos, $CdCl_2$, or H_2O_2

To examine the levels of GRP78 and HSP72/73 immunoreactive protein in RLE cells exposed to asbestos, $CdCl_2$ or H_2O_2, we performed Western blot analyses using antibodies specific for GRP78 and the constitutive (HSP73) and inducible (HSP72) forms of HSP72/73. Figure 2 shows levels of GRP78 immunoreactive proteins in RLE cells after 4, 8, 24, and 48 h of exposure to test agents. In comparison to controls, no striking increases in GRP78 protein levels are detected at any time point following exposure of cells to asbestos (5 and 10 μg/cm^2 dish) or H_2O_2 (200 μM). In contrast, after 48 h of exposure to $CdCl_2$ (10 μM), the levels of GRP78 protein were markedly increased (Fig. 2). Increases in HSP72/73 protein levels are also observed in RLE cells exposed to $CdCl_2$ (Fig. 3). This increase is evident as early as 8 h of exposure and remains elevated after 48 h of exposure. However, we observed no alterations in the levels of HSP72/73 protein in RLE cells exposed to crocidolite asbestos (5 and 10 μg/cm^2 dish) or H_2O_2 (200 μM) (Fig. 3).

Expression of cJun in RLE cells exposed to asbestos, $CdCl_2$, or H_2O_2

Previous results from our laboratory have shown that exposure of rodent tracheal epithelial and mesothelial cells to crocidolite asbestos causes a persistent increase in c-*jun* mRNA levels at periods from 8 to 24 h.[5,17,41] To relate GRP78 and HSP72/73 protein expression to an early-response gene product induced in cells after exposure to asbestos, we examined amounts of cJun in RLE cells following addition of asbestos, $CdCl_2$, or H_2O_2 (Fig. 4). Increases in cJun immunoreactive protein levels

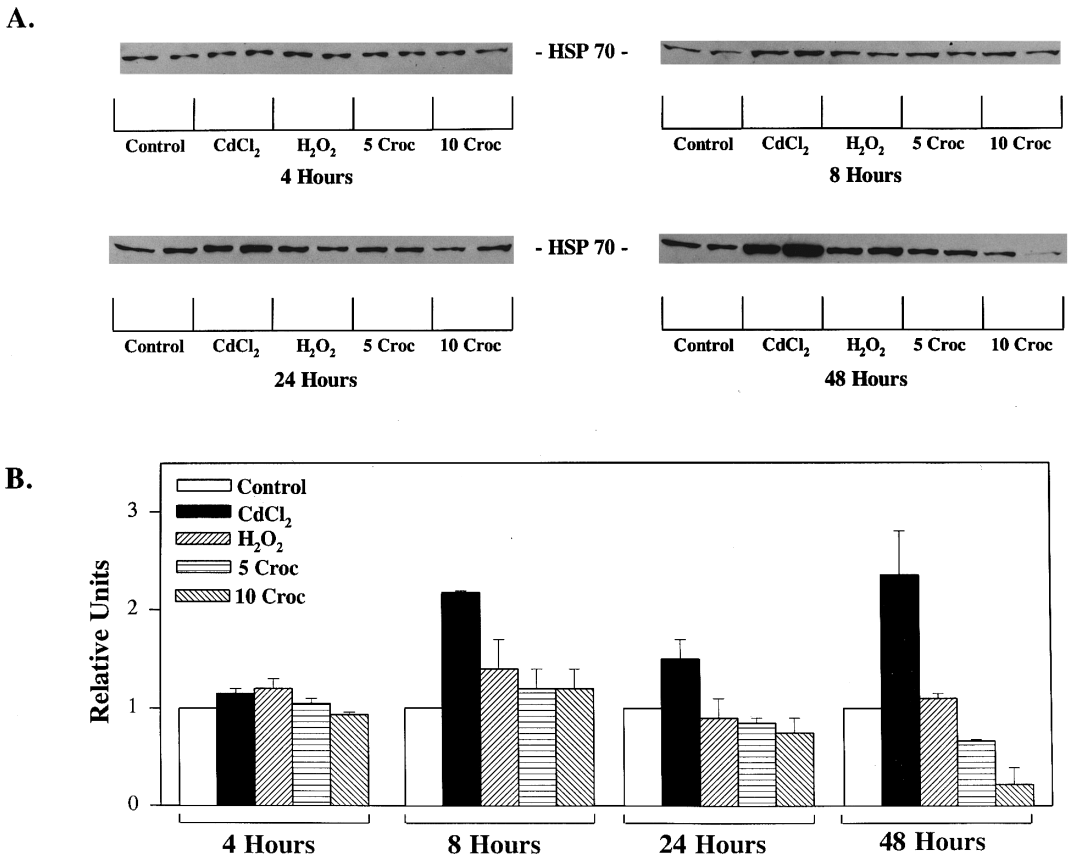

Fig. 3. Western blot analysis of HSP72/73 protein levels in RLE cells exposed to crocidolite asbestos, $CdCl_2$, or H_2O_2. Cell were cultured, exposed to test agents, and analyzed as described in Fig. 2. (A) Representative Western blot analysis of HSP72/73 protein levels in RLE cells exposed to test agents. (B) Densitometric analysis of HSP72/73 Western blot. Results are expressed as mean ± SEM.

were apparent at 24 and 48 h in cells exposed to 5 and 10 $\mu g/cm^2$ asbestos (Fig. 4). cJun protein levels were also elevated relative to unexposed controls in cells after 4 h of exposure to $CdCl_2$ or H_2O_2 (Fig. 4). By 24 h of exposure, levels of cJun protein in cells exposed to H_2O_2 decreased to control levels, but remained elevated in cells exposed to $CdCl_2$ at 48 h of exposure (Fig. 4).

Protein levels of MnSOD in RLE cells after exposure to crocidolite asbestos, $CdCl_2$, or H_2O_2

Increased levels of antioxidant enzymes in cells exposed to asbestos can serve as an indirect indicator of oxidant stress. To relate changes in GRP78 and HSP72/73 protein levels to patterns of oxidative stress, we examined the levels of MnSOD immunoreactive protein in RLE cells exposed to asbestos, $CdCl_2$, or H_2O_2. Figure 5 shows levels of MnSOD protein in RLE cells after 24 and 48 h of exposure to test agents. In comparison to controls, increases in MnSOD protein were observed at 24 and 48 h following exposure of cells to asbestos (5 and 10 $\mu g/cm^2$) and H_2O_2 (200 μM) (Fig. 5). In contrast, after 24 and 48 h of exposure to $CdCl_2$ (10 μM), the levels of MnSOD were unaltered.

Levels of GSSG and GSH in RLE cells exposed to asbestos, $CdCl_2$, or H_2O_2

To determine if exposure to agents alters thiol pools in RLE cells, we examined levels of GSSG and GSH in cells exposed to asbestos (5 and 10 $\mu g/cm^2$), $CdCl_2$ (10 μM) or H_2O_2 (200 μM). As can be seen in Fig. 6, no significant changes in GSH (Fig. 6A) were observed in RLE cells exposed to asbestos. Addition of H_2O_2 for 24 h resulted in increased GSH levels relative to untreated controls. In contrast, significant increases in GSH levels were observed in RLE cells after 24 and 48 h of exposure to $CdCl_2$ (Fig. 6A). A similar increase in GSSG levels was also observed at 24 and 48 h in RLE cells exposed to $CdCl_2$ (Fig. 6B). Increased GSSG levels were also observed in RLE cells after 24 h of exposure to

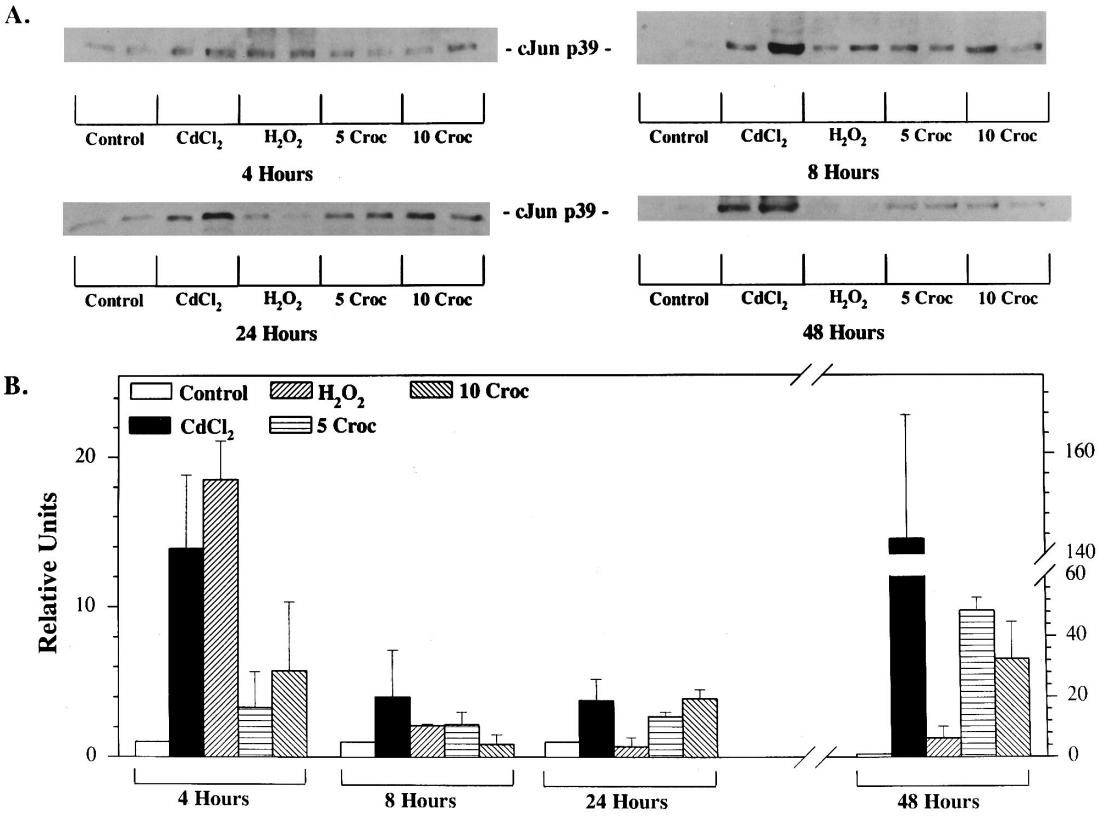

Fig. 4. Western blot analysis of cJun protein levels in RLE cells exposed to asbestos, $CdCl_2$, or H_2O_2. Cells were cultured, exposed to test agents, and analyzed as described in Fig. 2. (A) Representative Western blot of cJun protein levels in RLE cells exposed to test agents. (B) Densitometric analysis of cJun Western blot. Results are expressed as mean ± SEM.

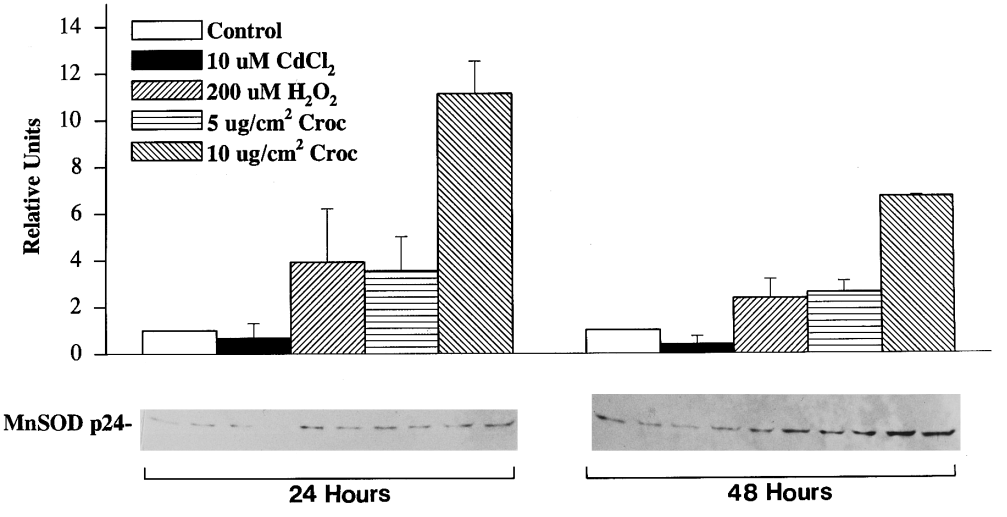

Fig. 5. MnSOD protein levels in RLE cells exposed to crocidolite asbestos, $CdCl_2$, or H_2O_2. (A) Western blot analysis of MnSOD protein levels after 24 and 48 h of exposure to asbestos (5 and 10 $\mu g/cm^2$), $CdCl_2$ (10 μM), or H_2O_2 (200 μM). (B) Densitometric analysis of MnSOD Western blot. Results are expressed as mean ± SEM.

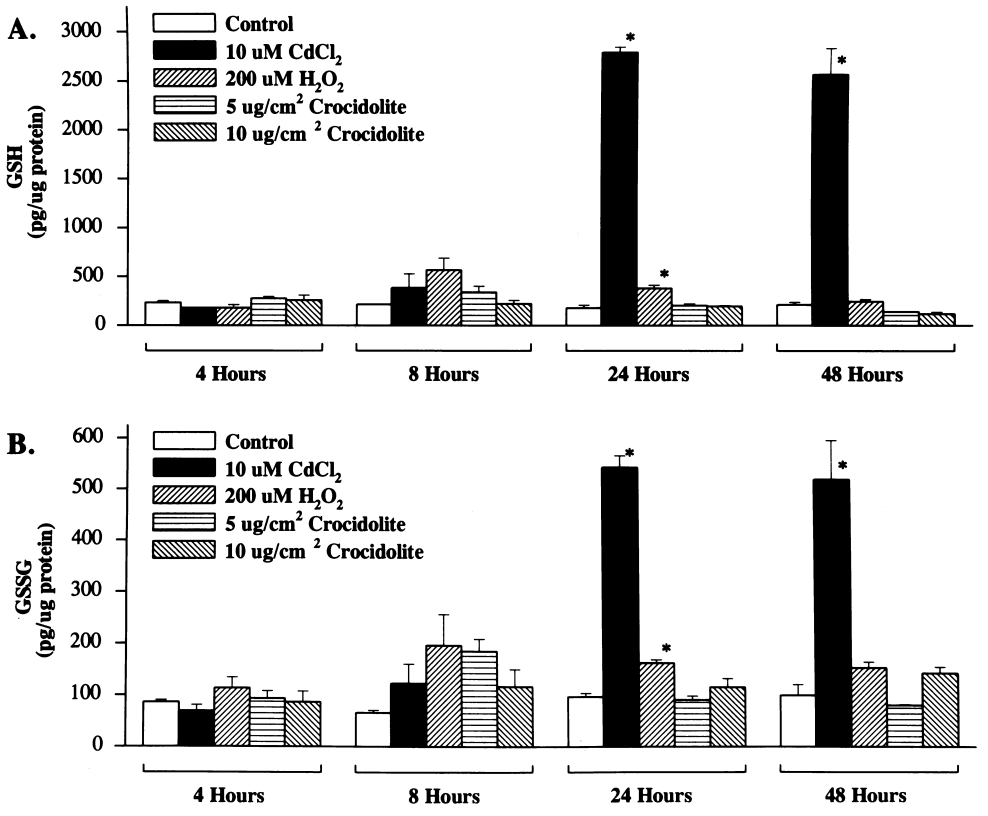

Fig. 6. GSH (A) and GSSG (B) levels in RLE cells exposed to crocidolite asbestos, $CdCl_2$, or H_2O_2. Results are expressed as mean ± SEM from a representative experiment. *$p < .05$.

H_2O_2, although no changes were seen at any time point in response to asbestos.

DISCUSSION

Mammalian cells respond to a diversity of environmental insults, both physical and chemical, by inducing the expression of a group of genes referred to as stress response genes. These include *grp*78,[18] *hsp*70,[19,27,28] and c-*jun*.[22,42] The induction of these genes and the functions of their encoded protein products define a defense mechanism(s) whereby cells detect damaged cellular components and activate appropriate scavenging and repair pathways. Although a number of environmental oxidants affect lung, studies have not focused on alterations of stress proteins by these agents or agent-specific effects in lung epithelium. We examined here protein expression of GRP78, HSP72/73, cJun, and MnSOD, an indication of oxidant stress, in target epithelial cells of lung after exposure to agents inducing oxidant injury.

Increased expression of GRP78 and HSP72/73 is considered to be a marker of proteotoxic stress, i.e., damage to cellular proteins. Because AOS are implicated as mediators of many of the events triggered by asbestos, we also examined protein levels of GRP78 and HSP72/73 following exposure to the oxidant, H_2O_2, and to the sulfhydryl reactive compound, $CdCl_2$. To compare changes in GRP78 and HSP72/73 protein levels to known markers of exposure to asbestos, we examined levels of cJun and MnSOD immunoreactive proteins. Our results show that exposure of RLE cells to cytotoxic doses of crocidolite asbestos does not alter the levels of GRP78 or HSP72/73 proteins. However, increased levels of the antioxidant enzyme, MnSOD, were observed indicating asbestos-induced oxidative stress in RLE cells.

Several forms of oxidative stress (e.g., UV irradiation, cadmium) have been shown to induce *hsp*70 gene expression.[33] In previous work by our laboratory as well as others, H_2O_2 has been used as a source of AOS to examine alterations in gene expression induced by oxidants. H_2O_2 can interact with intracellular iron to produce highly reactive hydroxyl radicals.[9] In RLE cells, exposure to H_2O_2 does not alter GRP78 or HSP72/73 protein levels. A similar finding was reported by Bruce et al.,[43] who demonstrated that exposure of NIH-3T3 cells to H_2O_2 failed to induce HSP72/73 gene expression or protein levels. However, H_2O_2 has been shown to induce the synthesis of heat-shock proteins in human

monocytes[44] and primary cultures of guinea pig gastric mucosal cells,[45] observations that may reflect cell-type specificity in the response to H_2O_2. We did observe increased levels of MnSOD protein in RLE cells exposed to H_2O_2 indicating a cellular response to this oxidant stress.

Cadmium mediates its toxic effects through a number of different mechanisms including interaction with protein sulfhydryls, production of AOS, and modulation of cellular GSH levels.[29] In RLE cells exposed to $CdCl_2$, we observed significant increases in the levels of GRP78 and HSP72/73 proteins, showing that increased levels of these stress response proteins is dependent on the inducing agent. Elevated GRP78 protein levels correlate with decreases in RLE cell viability indicating that cytotoxic doses of $CdCl_2$ are required to increase GRP78 protein. In contrast, $CdCl_2$-induced increases in HSP72/73 protein levels occur prior to loss of cell viability and may reflect an adaptive response of RLE cells to $CdCl_2$ toxicity. Other investigators have demonstrated induction of *hsp*70 gene expression after treatment with $CdCl_2$ in WISH cell cultures[29] and rodent models of cadmium-induced toxicity.[35] In addition to increased levels of HSP72/73, cadmium has also been shown to induce the expression of metallothionein, a small metal chelating protein that binds cadmium and may represent an important component in the adaptive response of cells to cadmium toxicity.[46,47]

The response of RLE cells to cytotoxic doses of asbestos is also characterized by increased levels of cJun protein. This observation is consistent with our previous studies demonstrating persistent increases in levels of c-*jun* mRNA in hamster tracheal epithelial and rat pleural mesothelial cells exposed to asbestos.[17] Elevated cJun protein levels are also observed in RLE cells exposed to H_2O_2 and $CdCl_2$ albeit with different patterns of expression. In RLE cells exposed to H_2O_2, cJun protein levels are elevated after 4 h of exposure and return to control levels by 24 h. In contrast, exposure of cells to $CdCl_2$ results in early increases in cJun protein that remain elevated at 48 h of exposure. Different patterns of cJun protein expression may reflect agent-specific responses in RLE cells exposed to asbestos, H_2O_2, or $CdCl_2$. Recently, basal levels of c-*jun* gene expression have been shown to be increased in H_2O_2- and O_2-resistant cell lines indicating a potential role for c-*jun* in the cellular response to oxidative stress.[48]

Glutathione, a major source of cellular protection against oxidative stress, functions as a scavenger of AOS and other free radicals.[49,50] Previous work in our laboratory has shown that exposure of rat pleural mesothelial cells to asbestos for 8 h (the time point of earliest induction of early-response protooncogenes by asbestos)[17] depletes total intracellular thiol pools, although GSSG and GSH were not measured comparatively.[41] Furthermore, asbestos-induced depletion of intracellular glutathione is associated with induction of the proto-oncogenes c-*jun* and c-*fos*[41] and apoptotic cell death (Jiminez et al., submitted) in pleural mesothelial cells. Pretreatment of mesothelial cell cultures with *N*-acetyl-L-cysteine, a glutathione precursor, blocks induction of c-*jun* and c-*fos*[41] and reduces apoptosis (Jiminez et al., submitted). In RLE cells exposed to asbestos, we did not detect changes in GSH or GSSG levels. The disparity in effects of fibers on intracellular thiol pools between epithelial and mesothelial cells may indicate cell-type specific responses to asbestos or differences in levels of antioxidants in these cells.

We observed similar trends of GSH and GSSG induction in RLE cells in response to H_2O_2 or $CdCl_2$. In comparison to $CdCl_2$, H_2O_2 caused less marked elevations in GSH and GSSG at 24 h, a result corresponding with increases in total thiol pools as previously reported in mesothelial cells.[41] In contrast, GSH and GSSG levels were markedly increased in RLE cells exposed to $CdCl_2$ at both 24 and 48 h. Elevated GSH pools in both H_2O_2 and $CdCl_2$-exposed RLE cells may represent a protective response to these agents. For example, increased GSH levels have been observed in Hep G2 cells exposed to $CdCl_2$[51] and are associated with resistance of A549 cells to $CdCl_2$-mediated toxicity.[52]

In summary, we have shown that oxidant-stress inducing agents, crocidolite asbestos, $CdCl_2$, and H_2O_2, elicit different patterns of stress protein expression in lung epithelial cells. Comparative studies examining the response of lung cells to asbestos and $CdCl_2$, a suspect lung carcinogen, or the oxidant, H_2O_2, may provide insight into the pathogenic mechanism(s) of each of these agents. Our observations support increasing evidence indicating that cellular responses to different toxic agents is specific and may be characterized by distinct patterns of stress protein expression. Knowledge of the response of pulmonary and pleural cells to asbestos and the mechanism(s) by which asbestos induces cellular damage will aid in identification of cellular response pathways that may have important implications for therapeutic approaches in asbestos-induced lung disease.

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