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Advances in Brief

Silica-induced Activation of c-Jun-NH_2-Terminal Amino Kinases, Protracted Expression of the Activator Protein-1 Proto-Oncogene, *fra-1*, and S-Phase Alterations Are Mediated via Oxidative Stress[1]

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Abstract

Crystalline silica has been classified as a group 1 human carcinogen in the lung. However, its mechanisms of action on pulmonary epithelial cells which give rise to lung cancers are unclear. Using a nontransformed alveolar type II epithelial cell line (C10), we show that α-quartz silica causes persistent dose-related increases in phosphorylation of c-Jun-NH_2-terminal amino kinases (JNKs) that are inhibited by antioxidants ($P \leq 0.05$). Increases in activator protein-1 (AP-1) binding to DNA and transactivation of AP-1-dependent gene expression by silica were accompanied by increases in steady-state mRNA levels of the AP-1 family members, c-*jun*, *jun*B, *fra*-1, and c-*fos* at 8 h and elevated mRNA levels of *fra*-1 at 24 h ($P \leq 0.05$). Addition of tetramethylthiourea inhibited silica-associated increases in *fra*-1 and proportions of cells in S-phase ($P \leq .05$). Our findings indicate that silica induces JNK activity, AP-1-dependent gene expression, *i.e.*, *fra*-1, and DNA synthesis via oxidative stress. Moreover, they suggest that silica may act mechanistically as a mitogen or tumor promoter, rather than a genotoxic carcinogen, in the development of lung cancers.

Introduction

Whether crystalline silica (SiO_2) is a carcinogen has been a subject of debate over the last decade, but an IARC working group concluded recently in a split vote that α-quartz, the most common silica polymorph compromising up to 95% of all sand and silt, is a group 1 human carcinogen in some occupational settings (1). This decision was based primarily on epidemiology and rat inhalation studies because silica does not appear to be genotoxic in a number of *in vitro* and *in vivo* assays assessing morphological transformation, gene mutations, and aneuploidy in mammalian cells, especially when the lack of appropriate controls, statistical significance, and dose-response data are considered (1). A more plausible mechanism of action in lung may be the stimulation of cell signaling cascades that trigger subsequent transcriptional events important in tumor development.

The MAPK[3] cascade consists of three arms including the ERKs, JNKs, and p38 kinases. Phosphorylation of these proteins may result in activation of a number of transcription factors that interact with regulatory domains in the promoter regions of genes that are integral to cellular responses such as proliferation, apoptosis, or inflammation. AP-1, comprised of members of the *jun* and *fos* proto-oncogene family, is a redox-sensitive transcription factor associated with the development of cell proliferation and tumor promotion (2).

In studies here, we used a murine alveolar epithelial type II cell line (C10; Ref. 3), as a cell type first encountering inhaled silica particles and a progenitor cell of lung cancers, to determine pathways of activation of MAPKs in relationship to AP-1 transactivation, expression of AP-1 family members, and functional outcomes, *i.e.*, changes in cell cycle distributions, after exposure to silica *in vitro*. Crocidolite asbestos, a documented carcinogenic fiber in mesothelial cells and tumor promoter in lung epithelium (4), was used as a positive control and GBs as a nonpathogenic particle (negative control). Our findings show protracted JNK activation, AP-1-dependent (*fra*-1) gene expression, and S-phase increases by silica via an oxidant-dependent mechanism. Moreover, they suggest that modification of cell signaling through antioxidant strategies may be valuable in preventive and therapeutic approaches to silica-induced lung cancers.

Materials and Methods

Cell Cultures and Addition of Particulates. The C10 cell line is a nontumorigenic murine alveolar type II epithelial cell line originally cloned from the NAL 1A alveolar type II epithelial cell line (3). The line was isolated from adult mice and maintains a characteristic epithelial morphology including surface microvilli, desmosomes, and lamellar bodies. C10 cells were maintained and passaged in CMRL 1066 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. At confluence, cells were switched to 0.5% fetal bovine serum-containing medium for 24 h prior to addition of α-quartz silica (Minusil) at final concentrations of 10 and 20 $\mu g/cm^2$ dish, crocidolite asbestos [$Na_2(Fe^{3+})_2(Fe^{2+})_3Si_8O_{22}(OH)_2$] (NIEHS reference sample) at 5 $\mu g/cm^2$ dish, or GBs (Particle Information Services, Inc., Kingston, WA) at 10 or 20 $\mu g/cm^2$ dish. All particulates were weighed and suspended at 1 mg/ml in HBSS (Life Technologies, Inc., Grand Island, NY) before addition at final noncytolytic concentrations to culture dishes (5, 6). Sham control and particulate-exposed cells were evaluated at time points from 1 h (the time required for sedimentation of particles onto cells) to 24 h thereafter.

Western Blot Analyses for Phosphorylated ERKs, JNKs, and p38 Kinases. In these experiments, H_2O_2 (300 μM for 30 min) was used as a positive control for phosphorylation of ERKs, p38, or JNKs (6). Cells were washed three times with cold PBS, collected in lysis buffer [20 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM Na_3O_4V, 10 mM NaF, 10 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride], vortexed for 1 min at 4°C, and centrifuged at 14,000 rpm for 20 min. The amount of protein in each sample was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Forty μg of protein in sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.1% w/v bromphenol blue] were electrophoresed in 10% SDS-polyacrylamide gels and transferred to nitrocellulose using a semidry transfer apparatus (Ellard Instrumentation, Ltd., Seattle, WA). Blots were blocked in buffer [TBS containing 5% nonfat powdered milk plus 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO)] for 1 h, washed three times for 5 min each in TBS/0.05% Tween 20, and incubated for 45 min with antibodies specific to phosphorylated

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[3] The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun-NH_2-terminal amino kinase; AP-1, activator protein-1; TMTU, tetramethylthiourea; GB, glass bead; NAC, *N*-acetyl-cysteine; H_2DCFDA, 2′,7′dichlorodihy-drofluorescein diacetate; TBS, Tris-buffered saline.

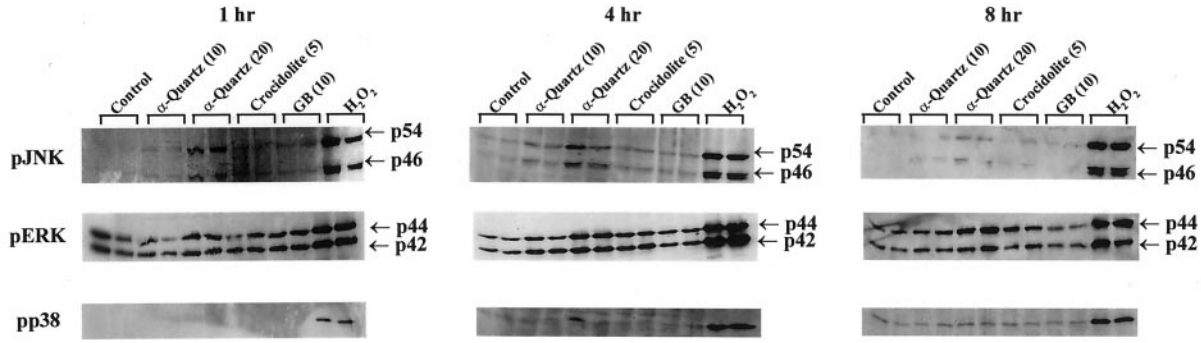

Fig. 1. Western blot analyses for phosphorylated JNKs, ERKs, and p38 kinases. Cells after exposure to particulates for 1, 4, and 8 h or H_2O_2 (positive control at 300 μM) for 30 min were lysed, proteins were electrophoresed in 10% SDS-polyacrylamide gels, and blots were incubated with phospho-specific antibodies before visualization of proteins using chemiluminescence. *Numbers* in *parentheses*, $\mu g/cm^2$ dish of particulates.

proteins [phospho-38 MAPK (Thr-180/Tyr-182 at 1:1000); phospho-p44/42 MAPK (Thr-202/Tyr-204 at 1:1000); phospho-JNK (Thr-183/Tyr-185) at 1:2000; New England Biolabs, Beverly, MA]. Blots then were washed three times with TBS/0.05% Tween 20 and incubated with an antirabbit peroxidase-conjugated secondary antibody for 30 min. After washing blots three times in TBS/0.05% Tween 20, protein bands were visualized with the LumiGlo enhanced chemiluminescence detection system (Kirkgaard and Perry Laboratories, Gaithersburg, MD).

JNK1 Activity Assay. The immunoprecipitation assay for JNK1 activity was performed as described previously using an anti-JNK1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:1000 dilution and GST-c-Jun (kindly provided by Dr. Roger Davis, University of Massachusetts, Worcester, MA) as a substrate for JNK1 (7). Incorporation of [γ-^{32}P]ATP into substrate was detected by autoradiography, and data were quantitated using a phosphorimager (Bio-Rad). In initial experiments to determine a role of antioxidants or iron-catalyzed oxidants in silica-induced JNK1 activity, cells were exposed to silica incubated in HBSS or HBSS containing the iron chelator, deferoxamine mesylate (Sigma; 1 mM) for 18 h (6). In other groups, catalase (500 units/ml; Sigma) was added to medium for 1 h (6), or cells were pretreated with NAC (10 mM) for 18 h (8) prior to exposure to silica for 1 or 4 h.

Preparation of Nuclear Extracts and Electrophoretic Gel Mobility Shift Assays for AP-1. Nuclear extracts of sham or particle-treated C10 cells were prepared as described previously (9). The amount of protein in each sample was determined using the Bio-Rad protein assay (Bio-Rad). One ng of a ^{32}P-end-labeled double-stranded oligodeoxynucleotide representing the fat-specific element that contains a TRE consensus sequence (*i.e.*, TGACTCA) was incubated with extract as described previously (10). The components of the AP-1 complex were identified by supershift analysis using antibodies specific for Fra-1, Fra-2, c-Fos, c-Jun, and JunD (data not shown). Autoradiograms were developed and quantitated using a Bio-Rad phosphorimager.

RNase Protection Assays. Confluent cultures maintained in 0.5% serum-containing medium for 24 h were exposed to particulates for 8 or 24 h. Total RNA was prepared and quantitated by absorbance at 260 nm (9). Steady-state mRNA levels of c-*jun*, *jun*B, *jun*D, c-*fos*, *fra*-1, *fra*-2, and *fos*B, the ribosomal

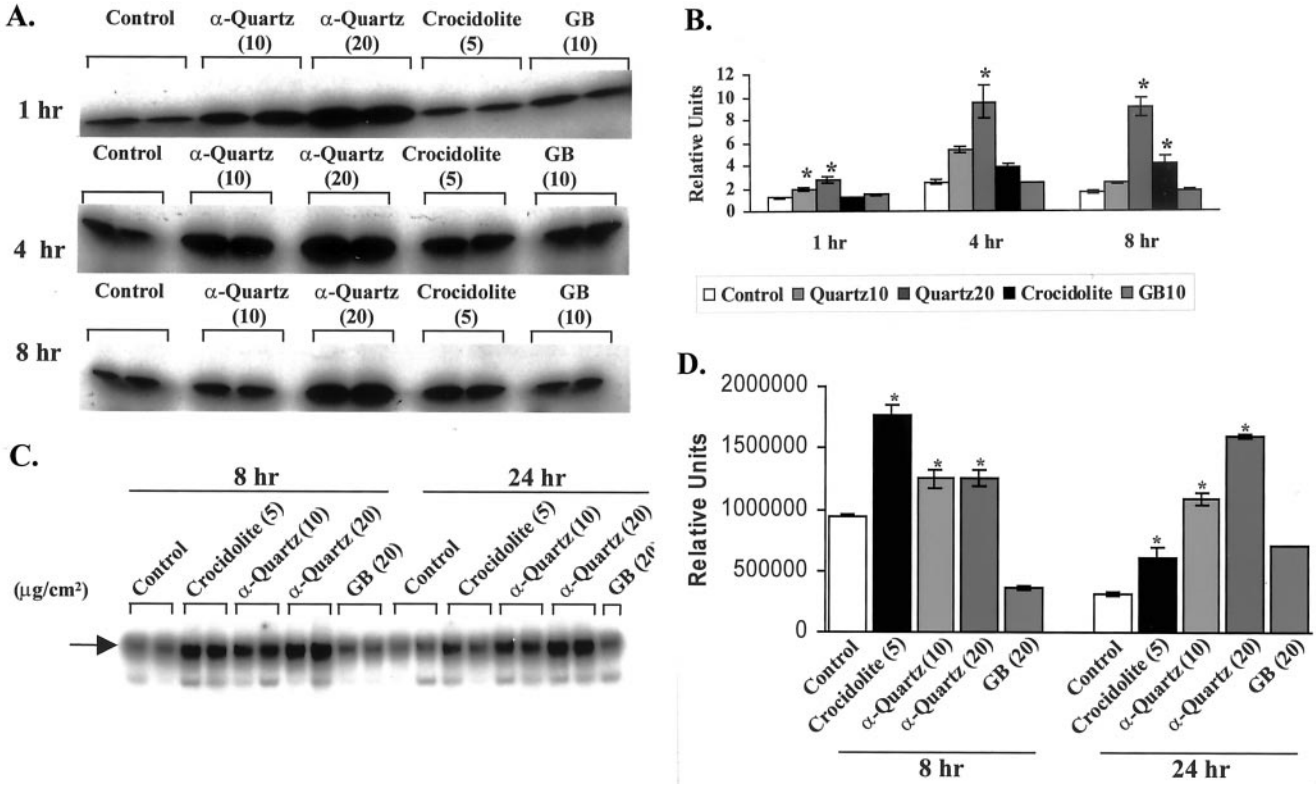

Fig. 2. Time frame and magnitude of increased JNK1 activity (*A* and *B*) and AP-1 binding to DNA (*C* and *D*) by α-quartz silica, crocidolite asbestos, and GBs in C10 cells. *A* and *C*, autoradiograms; *B* and *D*, quantitation of results by phosphorimaging. *Arrow* in *B*, the specific AP-1-fat-specific element DNA complex. *, $P \leq 0.05$, increased in comparison to sham control group at each time period. *Bars*, SE.

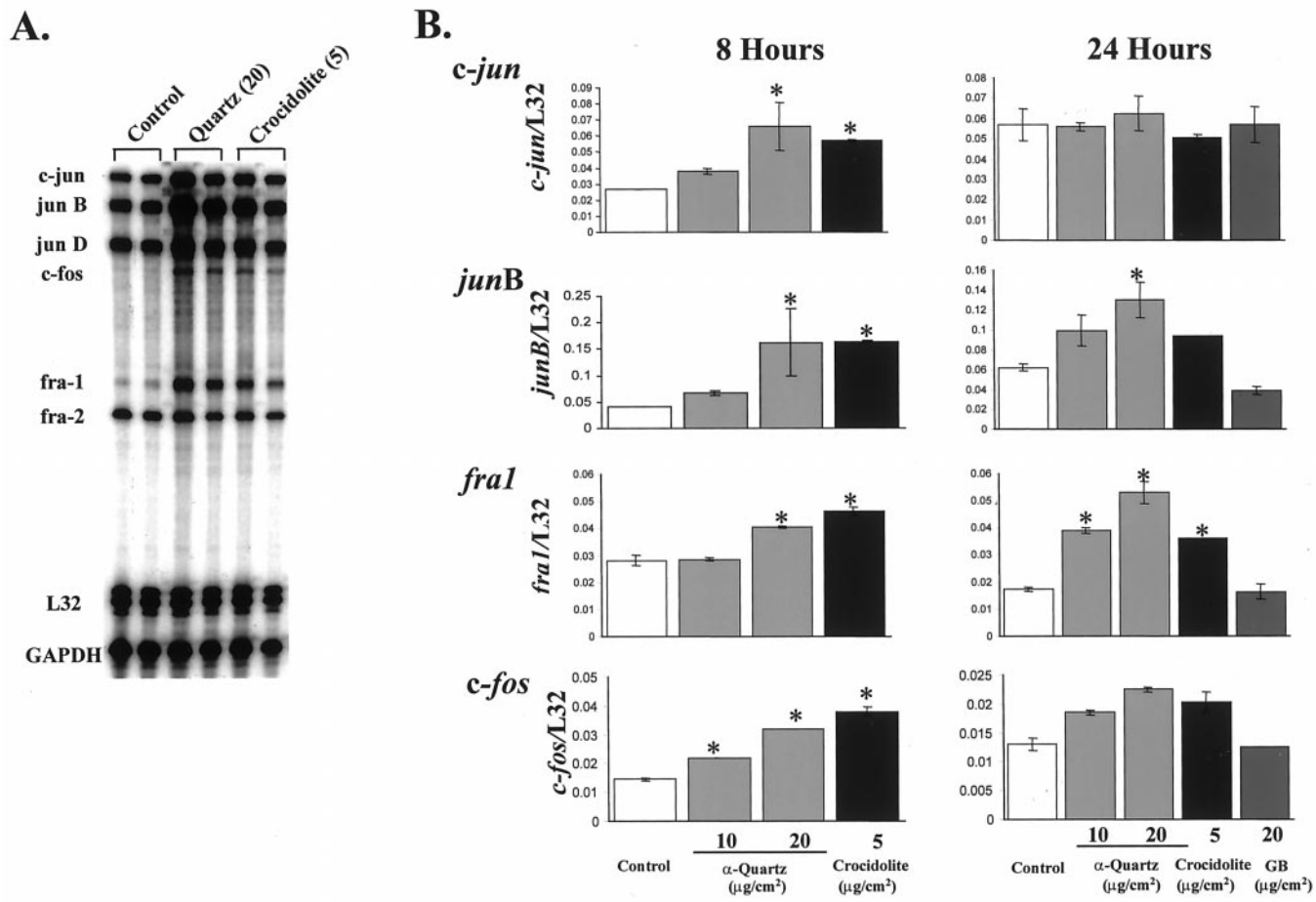

Fig. 3. RNase protection assay showing increased steady-state mRNA levels of AP-1 family members at 8 h and protracted expression of *fra*-1 after 24 h exposure to α-quartz silica or crocidolite asbestos. *A*, representative autoradiogram at 8 h. *B*, quantitation of results of duplicate experiments at 8 and 24 h using phosphorimaging. No changes in mRNA levels of *jun*B or *fra*-2 were observed (data not shown). ∗, $P \leq 0.05$ in comparison to sham control group at each time period. *Bars*, SE.

probe, L32, and glyceraldehyde-3-phosphate dehydrogenase were examined using the RiboQuant multiprobe RNase protection assay system and the mFos/Jun multiprobe template set (PharMingen, San Diego, CA) according to the manufacturer's protocol. Autoradiograms were quantitated using a Bio-Rad phosphorimager. Results were normalized to expression of the housekeeping gene, *L32*. In some experiments, the hydroxyl radical (OH·) scavenger, TMTU (25 and 50 mM), or catalase (500 units/ml medium; Ref. 6) was added 1 h prior to the addition of silica.

Flow Cytometry. Flow cytometry was used to determine alterations in cell cycle kinetics induced by particulates as described previously (11). At 24 h after addition of particles, cells were harvested by trypsinization, resuspended at 1×10^6 cells/ml in a propidium iodide staining solution (50 μg/ml propidium iodide, 0.1% Triton X-100, and 32 μg/ml RNase A in PBS), and incubated at 37°C for 30 min before analysis of 10,000 cells/group/time point in triplicate. The distribution of cells in the various cell cycle compartments, including cells with a hypodiploid DNA content indicative of apoptosis, was determined using a Coulter Epics Elite flow cytometer and appropriate software. Apoptosis was confirmed on C10 cells cultured on glass coverslips using an antibody recognizing single-strand DNA (Apostain; Alexis, San Diego, CA; data not shown).

Measurement of Intracellular Oxidant Accumulation. C10 cells at confluence were exposed to particles for 1, 8, or 24 h ($n = 3$/group/time point). After exposure to particles, H_2DCFDA (10 μM; Sigma), a nonfluorescent precursor of fluorescein, was added to each dish at a final concentration of 10 nM for 30 min at 37°C. Cells then were trypsinized, washed, and resuspended in HBSS without phenol red, and fluorescence was measured using a flow cytometer (Coulter Epics Elite; Coulter Corporation, Miami, FL) at excitation and emission wavelengths of 488 and 525 nm, respectively.

Statistical Analyses. All data were examined by ANOVA using the Student-Newman Keuls procedure to adjust for multiple pairwise comparisons between groups.

Results

Silica Causes Dose-related Increases in Phosphorylated JNKs and JNK Activity That Are Accompanied by Elevations in AP-1 Binding to DNA. The Western blot analyses in Fig. 1 show dose-related increases in phosphorylated JNK1/2 proteins at 1, 4, and 8 h after addition of silica. In contrast, exposure of C10 cells to asbestos or GBs failed to show elevations in JNK phosphorylation. Silica-associated increases in phosphorylated ERKs were also noted at 4 and 8 h and were seen as well with asbestos but not GBs. Changes in phosphorylated p38 were not observed after addition of minerals or GBs. Because prolonged increases in phosphorylated JNK proteins were a unique feature of silica (in contrast to GBs or asbestos), JNK1 activity assays also were performed. Data showed dose-related increases ($P \leq 0.05$) in response to silica at all time points (Fig. 2, *A* and *B*). At 8 h, a less striking but significant ($P \leq 0.05$) increase in JNK1 activity was also observed in cells exposed to asbestos. GBs failed to cause increases in JNK1 activity at any time point. Fig. 2, *C* and *D*, shows that AP-1 binding to DNA was increased ($P \leq 0.05$) at 8 and 24 h after exposure of C10 cells to silica (10 and 20 μg/cm^2). Elevations were also noted with addition of asbestos ($P \leq 0.05$) but not GBs at either time point. AP-1 transactivation by silica was confirmed in a stable C10 cell

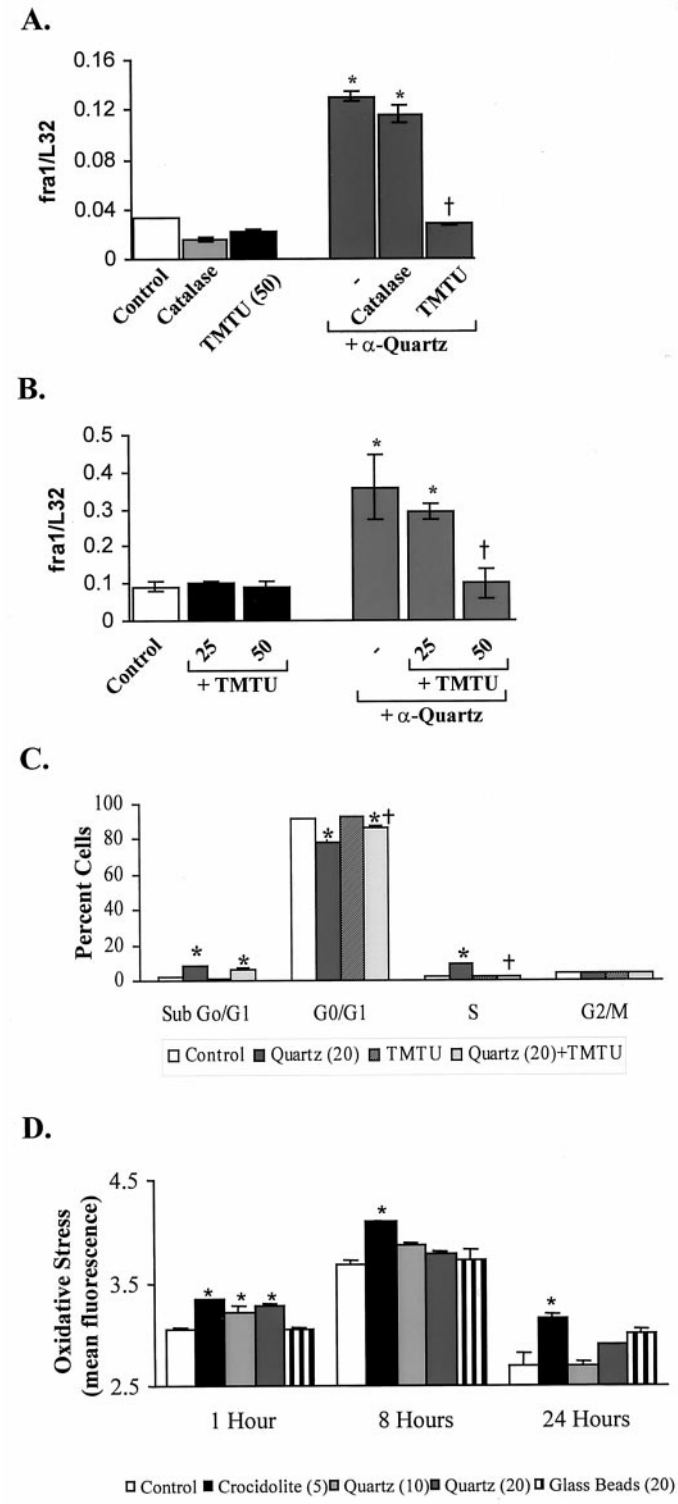

Fig. 4. The intracellular OH· scavenger, TMTU, inhibits mRNA levels of *fra*-1 and S-phase alterations by silica in C10 epithelial cells. *A* and *B*, results by phosphorimaging of RNase protection assay showing inhibition of α-quartz silica-induced mRNA levels of *fra*-1 by TMTU. *A*, cells were exposed to TMTU (25 or 50 mM) or catalase (500 units/ml) for 1 h prior to exposure to silica for 8 h. *B*, dose-response experiment using TMTU at 25 and 50 mM. *C*, proportions of C10 cells in various stages of the cell cycle after exposure to α-quartz silica alone or pretreated with TMTU (25 mM) for 1 h. Cells were harvested by trypsinization, stained with propidium iodide, and analyzed at 24 h using a flow cytometer. *, $P \leq 0.05$ in comparison to sham control groups at each time point. *D*, intracellular accumulation of oxidants as detected using H_2DCFDA and flow cytometry. Cells at 1, 8, and 24 h after addition of particulates were examined by flow cytometry after addition of H_2DCFDA (10 nM) for 30 min at 37°C, trypsinization, and resuspension in HBSS. Data show the means/group ($n = 3$); *bars*, SE. *, $P \leq 0.05$ in comparison with sham control groups at each time period. †, $P \leq 0.05$ in comparison with the group exposed to silica alone.

line expressing the reporter plasmid, *jun*-luciferase (7). In these experiments, both silica at 20 μg/cm^2 and asbestos (5 μg/cm^2 dish) caused significant increases in luciferase activity ($P \leq 0.05$), whereas GBs were inactive (data not shown).

Silica Causes Protracted Increases in mRNA Levels of *fra*-1. RNase protection assays were used to determine whether increased steady-state mRNA levels of *jun* and *fos* family members were altered after exposure to silica (Fig. 3). At 8 h, increases in mRNA levels of c-*jun*, *junB*, *fra*-1, and c-*fos* were significantly increased ($P \leq 0.05$) after exposure to silica at 20 μg/cm^2, and elevations ($P \leq 0.05$) in c-*fos* levels also were observed at lower concentrations of silica (10 μg/cm^2 dish). Significant increases ($P \leq 0.05$) in *fra*-1 mRNA at both concentrations of silica as well as elevations in mRNA levels of *junB* at 20 μg/cm^2 silica persisted for 24 h. Similar patterns of early *jun*/*fos* and more protracted *fra*-1 gene expression were also observed after addition of asbestos. GBs failed to cause any changes in gene expression.

Increases in *fra*-1 mRNA Levels by Silica Are Inhibited Using the Intracellular OH· Scavenger, TMTU. We initially examined the effects of a number of antioxidants on silica-induced JNK1 activity. Cells exposed to deferoxamine-treated silica or cells pretreated with NAC (10 mM) or catalase (500 units/ml) failed to show significant elevations in JNK activity by silica (data not shown).

On the basis of these results, we pre-exposed C10 cells to TMTU (25 and 50 mM), which can scavenge intracellular OH·, or catalase (500 units/ml medium), an extracellular scavenger of H_2O_2, for 1 h in an attempt to determine whether silica-induced increases in *fra*-1 could be altered. In comparison to sham control groups, no changes in gene expression were observed after addition of TMTU or catalase alone to cells (Fig. 4, *A* and *B*). However, addition of TMTU at 50 mM, but not catalase, to silica-exposed cells caused significant decreases ($P \leq 0.05$) in mRNA levels of *fra*-1.

Silica Causes Increases in the Proportion of C10 Cells in S-Phase That Are Inhibited by TMTU. To determine whether exposure of C10 cells to silica (10 and 20 μg/cm^2) resulted in alterations in cell cycle distributions, we performed flow cytometry using propidium iodide staining. These studies revealed that silica at 10 μg/cm^2 caused significant increases ($P \leq 0.05$) in the proportions of cells in S and G_2-M phases, whereas higher exposures (20 μg/cm^2) resulted in elevations ($P \leq 0.05$) of cells in sub-G_0-G_1 and S-phase. These were confirmed as apoptotic cells using the Apostain technique (data not shown). No changes in cell cycle distributions were observed after addition of GBs to cultures, whereas asbestos caused striking increases ($P \leq 0.05$) in the percentage of cells in sub-G_0-G_1 (data not shown). The addition of TMTU alone did not alter cell cycle kinetics nor increased numbers of cells in sub-G_0-G_1 when added prior to silica. However, the percentage of silica-exposed cells in S-phase was significantly inhibited by TMTU ($P \leq 0.05$; Fig. 4*C*).

To confirm that silica causes increased production of intracellular oxidants by pulmonary epithelial cells, intracellular levels of the fluorescent product of H_2DCFDA, an indicator of oxidant production, were measured. As shown in Fig. 4*D*, oxidant accumulation was significantly elevated ($P \leq 0.05$) in C10 cells exposed to silica (10 and 20 μg/cm^2) for 1 h. In contrast to silica, asbestos (5 μg/cm^2) caused protracted increases in oxidant production over a 24-h period. GBs (20 μg/cm^2) were inactive at all time points.

Discussion

Studies here show how silica, which is associated in some worker populations with an increased incidence of lung cancers, primarily in smokers (1), may act mechanistically as a nongenotoxic agent or tumor promoter in lung. We demonstrate early and protracted in-

creases in JNK1/2 phosphorylation and JNK1 activity that are unique to silica and associated with subsequent increases in message levels of *fos*/*jun* family members, their presence in AP-1 complexes, and transactivation of AP-1-dependent gene expression. Substrates for JNKs include ATF2, p53, and c-*jun*, proteins implicated in proliferation and/or apoptosis in various cell models (2). Overexpression of c-*jun* in tracheal epithelial cells enhances cell proliferation and transformation (7), suggesting a role of c-Jun and AP-1 transactivation in mitogenesis. These results are supported by immunocytochemistry showing increased c-Jun protein in hyperplastic and metaplastic epithelial cells from patients with lung cancers (12). Recently, we have shown that ambient airborne particles induce elevations in JNK activity and levels of phosphorylated c-Jun protein in rat pulmonary epithelial cells at lower concentrations, causing increased DNA synthesis, but not at high concentrations where apoptosis is observed (13). On the basis of the results summarized above, increased and persistent JNK activity by silica might reflect or be causally related to subsequent increases in proportions of C10 cells in S-phase. We also show the modulation of silica-induced JNK activity by intervention with antioxidants, suggesting that early signaling alterations by silica can be altered. The observation that silica-induced JNK activity can be prevented by catalase, a large molecule that cannot cross the cell membrane, or pretreatment of cells with NAC or deferoxamine, an iron chelator, suggest that both extracellular and intracellular generation of oxidants by silica may trigger JNK activation.

Another novel finding here is the increased expression of *jun* and *fos* proto-oncogenes in pulmonary epithelial cells exposed to silica. These studies support our hypothesis that pathogenic particles elicit signal transduction cascades that lead to the increased expression of early-response genes and AP-1 transactivation (4, 10). For example, increased mRNA levels of c-*jun* are observed in rodent lungs after inhalation of crocidolite asbestos, which accompany early increases in proliferation of epithelial cells (14).

In our studies, increased mRNA levels of *fra*-1 and *junB* persisted for 24 h. Moreover, Fra-1 and JunB were observed in AP-1 complexes from silica-exposed cells (data not shown). The significance of various AP-1 family members that comprise homo- and heterodimers that may govern AP-1 binding/stability, transactivation and subsequent alterations in gene expression is largely unknown, but some studies provide insight into functional roles of *fos* and *jun* family members. For example, as summarized above, overexpression of c-*jun (*which was elevated as well as c-*fos* in our studies after addition of silica or asbestos) in tracheal epithelial cells confers an increase in cell proliferation and morphological transformation (7). *jun*B and *jun*D attenuate c-*jun* activity and suppress proliferation, respectively, in other cell models (15, 16). Thus, a balance between *fos* and *jun* family members may govern the outcomes of increased DNA synthesis and apoptosis observed in silica-exposed epithelial cells. Recent studies suggest a critical role of Fra-1 in the development of epithelial tumors, because expression of exogenous Fra-1 in epithelioid adenocarcinoma cells results in increased motility and invasiveness (17). Overexpression of Fra-1 in rat fibroblasts causes anchorage-independent growth and tumors in athymic mice as well as up-regulation of its own gene (18). Moreover, constitutive expression of c-Fos, FosB, or c-Jun in these cells leads to AP-1-dependent stimulation of *fra*-1 expression.

Most notably, increases in *fra*-1 and *junB* mRNA levels by silica were inhibited significantly after addition of TMTU, an OH· scavenger, to C10 cells. Because catalase did not inhibit their expression, intracellular generation of oxidants, as verified in our studies, is implicated in silica-induced increases in *fra*-1. These data are exciting because they provide a rationale for modulation of silica-induced proto-oncogene expression by antioxidants. In summary, results here show induction of JNK activity and proto-oncogene expression by silica via an oxidant-dependent mechanism. Increases in *fra*-1 and *junB* mRNA levels may be causally related to increased AP-1 activity and cell cycle changes in pulmonary epithelial cells exposed to silica. Thus, approaches to alter these cell signaling events may be beneficial in altering cell proliferation in silica-associated cancers and fibroproliferative lung diseases.

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