Mechanisms Associated with Human Alveolar Macrophage Stimulation by Particulates

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Asbestos and silica are well-known fibrogenic dusts. However, there is no comprehensive understanding of the molecular and cellular events that lead to fibrosis as a consequence of asbestos or silica inhalation. Previous studies have shown that asbestos stimulates superoxide anion production in alveolar macrophages through the phospholipase C/protein kinase C pathway. In contrast, silica does not appear to activate this pathway nor stimulate superoxide anion production, but silica does stimulate cytokine release by some undetermined pathway. Therefore, using human alveolar macrophages isolated from normal healthy volunteers, we evaluated the potential involvement of intracellular calcium and tyrosine kinases as potential signal transduction pathways. In the absence of serum, crystalline silica, and to a lesser extent amorphous silica, caused a rapid and dose-dependent elevation of intracellular calcium coming from the extracellular space. However, in the presence of serum, which is required for silica-stimulated cytokine release, neither form of silica caused noticeable elevation of intracellular calcium. Silica, however, did increase the extent of tyrosine phosphorylation, most notably of proteins at approximately 46 and 50 kDa, suggesting activation of a tyrosine kinase pathway. Preincubation of alveolar macrophages for 24 hr with silica-primed human alveolar macrophages for enhanced interleukin-1β (IL-1β) release stimulated by endotoxin (LPS) that was dose dependent. The enhanced LPS-stimulated release of IL-1β correlated with enhanced mitogen-activated protein kinase activity. Taken together, these results indicate that a tyrosine kinase pathway is activated during silica stimulation of human alveolar macrophages. — Environ Health Perspect 102(Suppl 10):69–74 (1994)

Key words: human alveolar macrophage, intracellular calcium, MAP kinase, tyrosine kinase, silica

Introduction

Of all inhaled particulates, asbestos and silica are unique in their ability to cause lung fibrosis. Both are recognized as ubiquitous occupational fibrogenic agents, capable of inducing fibroblast proliferation and excess collagen production in the lung (1). Although asbestos- and silica-induced lung fibrosis are relatively well described on an anatomic level, there is as yet no comprehensive understanding of the molecular and cellular events that lead to fibrosis as a consequence of asbestos or silica inhalation.

The alveolar macrophage occupies a key position in mediating the interaction between inhaled particulates and other lung cell types, such as lymphocytes and fibroblasts. In addition to its abilities to chemotax and phagocytose, the alveolar macrophage produces a wide variety of inflammatory and growth-mediating factors such as superoxide anion and cytokines (2,3). The alveolar macrophage is therefore hypothesized to play a central role in the development of particulate-induced pulmonary fibrosis (4–6) through the inappropriate release of effector molecules with bioactivity for lung cells in response to fibrogenic particulates.

Alveolar macrophages from animals (7,8) and healthy human volunteers (9) exposed to fibrogenic particulates in vitro release factors that enhance fibroblast growth. Consistent with these in vitro observations, it has been shown that alveolar macrophages recovered from animals exposed to fibrogenic particulates (10) and from individuals with interstitial lung disease (11) caused by asbestos, silica, or coal dust appear to be in an activated state, as reflected by their enhanced basal production of cytokines such as interleukin-1β (IL-1β). A recent study indicates that basal levels of alveolar macrophage cytokine production are also elevated in those human subjects who have had extensive exposure to asbestos but who have no evidence of fibrosis (12). Macrophages from these latter subjects were found to release elevated amounts of several effectors, including IL-1β, tumor necrosis factor-α (TNF-α), interleukin-6, granulocyte-macrophage colony stimulating factor, and prostaglandin E2 (12). Whether the release of these factors by alveolar macrophages exposed to particulates in vivo is a result of their direct interaction with the particulates, an indirect result of the interaction of the particulates with other lung cell types, or both, is not known.

Previous in vitro studies using alveolar macrophages isolated from guinea pigs and humans demonstrated that while chrysolite and crocidolite asbestos stimulate superoxide anion production, silica was not bioactive for the release of this effector molecule (13). Furthermore, the pathway for asbestos stimulation of alveolar macrophage superoxide anion production involves the phospholipase C/protein kinase C pathway (14) and opening of verapamil-sensitive calcium channels (15). In contrast, both chrysolite and silica stimulated cytokine release (e.g., TNF-α and IL-1β) indicating either that the mechanism of silica stimulation was distinct from chrysolite or that chrysolite stimulated multiple pathways (16).

Results from other laboratories using rodent alveolar macrophages indicate that silica can cause a marked elevation of intracellular calcium in vitro, implicating it as a potential intracellular signal (17,18). Therefore, in the present study we examined whether silica caused an elevation of intracellular calcium in human alveolar macrophages under similar conditions that are required for cytokine release. In addition, the potential involvement of tyrosine kinases in silica stimulation was also evaluated.

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Materials and Methods

Particulates

Particulates for these studies included chrysotile asbestos and silica, and the nonfibrogenic particulate titanium dioxide. Particulate concentrations were chosen to represent equivalent surface areas. Unless otherwise noted, particulates and their concentrations used in these studies were Calidria asbestos RG-144 (Union Carbide, King City, CA), natural length 1–5 μm, 25 μg/ml; crystalline silica, min-U-sil 5 (Pennsylvania Sand Glass Corp., Pittsburgh, PA), 1–5 μm, 133 μg/ml; amorphous silica (Sigma Chemical Co., St. Louis, MO), 1–3 μm, 80 μg/ml; and titanium dioxide (Particle Information Services, Kingston, WA), average particle diameter 0.45 μm, 60 μg/ml.

Human Alveolar Macrophages

Cells for these experiments were obtained by bronchoalveolar lavage of normal non-smoking volunteers using methods described previously (19). Typically, instillations of 240 to 300 ml of sterile saline resulted in recoveries of 200 to 260 ml of lavage fluid. Cells were isolated from the lavage fluid as described previously (13), and kept on ice in HEPES-buffered Medium 199 (Whittaker Biochemicals, Walkersville, MD) with or without 10% heat-inactivated fetal calf serum (FCS, Sigma), at a concentration of 1 x 10⁶ cells/ml (ZBI, Coulter Counter, Coulter Electronics, Hialeah, FL) until use. Lavages yielded an average of 10% cells that were >92% macrophages as identified using Leukostat (Fisher Scientific, Fairlawn, NJ) staining and confirmed by esterase staining (20) of cytocentrifuge preparations. Cells were >85% viable by Trypan blue exclusion.

Intracellular Calcium

Cytosolic calcium concentrations were monitored continuously using the fluorescent probe Fura-2, similar to that previously described (15). Macrophages (10⁶/ml) were loaded for 30 min with 5 μM Fura-2 (Molecular Probes, Eugene, OR) in Medium 199 at 25°C. The cells were washed three times and resuspended in the same medium. Cells were placed in a continuously stirred cuvette with or without 10% FCS at 37°C in a Photocell Technology Inc. Delta Scanner dual wavelength fluorimeter with excitation at 340 and 380 nm and emission at 511 nm. Fluorescence intensities were continuously recorded after addition of silica and stored on a computer. The fluorescence data was converted to calcium concentrations using the equation (21):  

\[ [\text{Calcium}] = K_d (F - F_{\text{min}})/(F_{\text{max}} - F), \]

where \( K_d \) = 224 nM, \( F \) = relative level of intracellular fluorescence, \( F_{\text{min}} \) = relative level of fluorescence with 10 mM EGTA after cell lysis with 0.1% Triton X-100, and \( F_{\text{max}} \) = relative level of fluorescence in the presence of 4 mM calcium.

 Cultures for IL-β Release

Cells (1 x 10⁶/ml) were cultured for 24 hr in the presence or absence of 33 or 133 μg/ml silica in medium 199 with 10% FCS. After 24 hr, lipopolysaccharide (LPS) at 10 μg/ml or 100 ng/ml was added to stimulate human alveolar macrophage IL-β release for 24 hr. Cells were maintained in suspension by slow end-over-end tumbling in polypropylene tubes at 37°C. Incubations were terminated by pelleting the cells, and supernatants were frozen and stored at –80°C until assayed. IL-1β released into the supernatants was quantitated by EIA (Cayman Chemical, Ann Arbor, MI).

Immunoblotting for Phosphotyrosine

Cells were washed in phosphate-buffered saline, denatured and subjected to SDS polyacrylamide gel electrophoresis. Fractionated proteins were transferred to nitrocellulose membranes by electroblotting. Membranes were stained with 2% Ponceau S (Sigma) to visualize protein transfer to the membranes. Membranes were incubated in blocking buffer (5% nonfat dry milk, Tris-buffered saline) at 16 hr at 4°C, and then with antiphosphotyrosine monoclonal antibody (Upstate Biochemical Inc. (UBI), Upstate, NY, clone 4G10) for 2 hr at room temperature. The membranes were rinsed with Tween 20 in Tris-buffered saline and incubated with horseradish peroxidase-conjugated goat antimonospecific immunoglobulin G (Amersham, Arlington Heights, IL) for 1 hr at room temperature. After rinsing with Tween 20 in Tris-buffered saline, the antibody-antigen complex was visualized by using enhanced chemiluminescence detection (Amersham) and by exposing the membranes to ECL film (Amersham) for 1 min.

Mitogen-activated Protein (MAP) Kinase Activity

The procedure was adapted from Clark-Lewis et al. (22). At various times after adding LPS, cells were solubilized for 30 min on ice in 50 mM Tris-150 mM saline buffer, pH 8.0, containing 1% Nonidet P-40, 1 mM EDTA, 100 μM sodium orthovanadate, 1 μg/ml each of pepstatin and leupeptin, 0.1% aprotinin, and 2 mM phenylmethylsulfonyl fluoride. The solubilized fraction was collected following centrifugation and divided into two aliquots. To the solubilized proteins, antihuman MAP kinase (UBI) was added to a final concentration of 1 μg/ml and incubated for 1 hr, then an equal volume of Protein A agarose (10% suspension in the same buffer) was added and incubated for another hour. The agarose beads were washed three times in the same buffer and then used for the kinase assay. For the kinase assay, 25 μl of reaction cocktail was added to the agarose beads that consisted of 2 mg/ml of MAP kinase substrate, APRTGGR (UBI), 1 μM ATP with 2 μCi/sample of [γ-32P]ATP, 12.5 mM MOPS (pH 7.2), 12.5 mM glycerol phosphate, 7.5 mM MgCl₂, 0.5 mM EGTA, 0.05 mM NaF, 0.5 mM sodium orthovanadate, and 2 mM dithiothreitol. The reaction was allowed to proceed for 15 min and stopped by spotting the entire contents onto 2.1-cm circles of phosphocellulose paper. The papers were washed seven times with 1% phosphoric acid and the bound radioactivity determined by liquid scintillation counting. Negative controls included an irrelevant antibody.

Endotoxin

Endotoxin levels in buffers, media, and particulate stock suspensions were assessed by the Limulus amoebocyte assay (Sigma); endotoxin levels were in all cases found to be <20 pg/ml.

Statistics

Values are presented as the mean, and error bars denote the standard error of the mean. The number of individuals from whom cells were obtained for a given experiment is denoted by "N" in the corresponding figure legend. For measurements of IL-1β, statistical treatment was one-tailed paired t-test.

Results

Silica-induced Changes in Intracellular Calcium

Previous studies using rodent alveolar macrophages have shown that silica can cause an elevation in intracellular calcium in vitro (17,18). Since elevation in intracellular calcium could serve to signal downstream events, the effect of silica on intracellular calcium in human alveolar
macrophages using Fura-2 fluorescence was examined. As shown in Figure 1, crystalline silica caused a rapid and sustained elevation of intracellular calcium. Addition of 67 or 133 μg/ml silica caused intracellular calcium to increase from a baseline of approximately 150 nM to approximately 300 nM or almost 500 nM in approximately 9 min, respectively. In contrast, intracellular calcium remained constant in untreated cells. Furthermore, there was no change in intracellular calcium following silica addition to cells when 4 mM EDTA was added just prior to silica, indicating that the elevation of intracellular calcium was dependent on extracellular sources of calcium.

Similar results were obtained using amorphous silica (Figure 2). Amorphous silica is generally considered less fibrogenic than crystalline silica (23). Consistent with decreased fibrogenic potential there was decreased ability to elevate intracellular calcium compared to crystalline silica when given at approximately equal surface areas, i.e., 40 and 80 μg/ml (to levels of approximately 200 and 300 nM over the same time period, respectively). In addition, elevation of intracellular calcium with amorphous silica was also blocked using EDTA and therefore dependent on extracellular calcium.

The above experiments were conducted in the absence of added serum. However, in the absence of serum, silica does not stimulate human alveolar macrophages to release cytokines (data not shown). In the presence of serum, silica stimulates the release of a number of cytokines including IL-1 (16). Therefore, the ability of silica to elevate intracellular calcium under conditions required for cytokine release were also examined. As shown in Figure 3, neither 133 μg/ml crystalline nor 80 μg/ml amorphous silica caused noticable elevation of intracellular calcium in the presence of 10% FCS.

### Silica-induced Activation of Intracellular Tyrosine Kinases

The above results suggest that under conditions required for cytokine release silica stimulation of human alveolar macrophages may proceed by a pathway independent of changes in intracellular calcium. Other studies indicated that silica also does not cause noticable changes in cAMP or cGMP levels (data not shown). However, recent studies from other laboratories show that tyrosine kinases are involved in endotoxin stimulation of neutrophils and mitogen stimulation of lymphocytes (24,25).

Therefore, the potential involvement of tyrosine kinases in silica stimulation was examined by Western blot analysis using an antiphosphotyrosine antibody. Results of phosphotyrosine Western blots are shown for both 100 ng/ml LPS (Figure 4A) and 133 μg/ml crystalline silica (Figure 4B) stimulation of human alveolar macrophages as a function of time. Both LPS and silica caused rapid (<7.5 min) increased phosphorylation of tyrosine residues on specific proteins at approximately 46 and 80 kDa by LPS and 46 and 50 kDa by silica.

### Silica Priming of Human Alveolar Macrophages

Preliminary studies in our laboratory suggested that silica could prime alveolar macrophages for an enhanced response to LPS. Of a number of particulates that were examined, including chrysotile, titanium dioxide, and amorphous silica (data not shown), only crystalline silica was able to prime human alveolar macrophages for enhanced IL-1β release in response to LPS stimulation, as shown in Figure 5. The response was also dose dependent with silica and LPS. Enhanced IL-1β release was
Figure 3. Effect of serum on silica-induced elevation of intracellular calcium levels in human alveolar macrophages. Cells were incubated in the presence of 10% serum prior to addition of silica. The figure shows continuous recordings of intracellular calcium for cells incubated with 133 μg/ml crystalline silica (1), or 80 μg/ml amorphous silica (2), and the center trace is control. The arrow indicates the time of silica addition. The traces are representative of two experiments.

Figure 4. Tyrosine phosphorylation following endotoxin and crystalline silica stimulation of macrophages. Tyrosine phosphorylation was detected as described in "Materials and Methods" for macrophages stimulated with (A) 100 ng/ml endotoxin (B) or 133 μg/ml silica. The time of sampling is shown in minutes following addition of either stimulant. The results are representative of three experiments.

Discussion

Both silica and chrysotile asbestos stimulate human alveolar macrophages. However, while both particulates stimulate cytokine release (16), only asbestos stimulates superoxide anion production (13). These observations imply that either asbestos stimulates multiple pathways and one of them is similar to that stimulated by silica, or that the particulates stimulate independent pathways. We have already described that asbestos stimulates the phospholipase C/protein kinase C pathway (14) and opens a verapamil-sensitive calcium channel in alveolar macrophages (15). Other investigators using rodent alveolar macrophages have demonstrated that silica can cause an elevation of intracellular calcium (17,18). Our results with human alveolar macrophages are consistent with those reports in that crystalline silica and, to a lesser extent, amorphous silica can rapidly cause elevation of intracellular calcium (Figures 1, 2). Furthermore, the source of the increase in intracellular calcium appears to be from the extracellular pool, since it could be blocked by removal of extracellular calcium. The mechanism by which calcium enters macrophages is not certain, but may involve the opening of calcium channels.
since preliminary experiments indicate that it could be blocked by verapamil (unpublished results).

Different results were obtained when alveolar macrophages were incubated in the presence of 10% FCS prior to the addition of silica. In the presence of serum, neither form of silica was able to noticeably elevate intracellular calcium (Figure 3). Although silica may elevate intracellular calcium at a later time point, there is a clear alteration in bioactivity when serum is present, consistent with previous observations that the bioactivity of silica (macrophage superoxide anion production) can be modified significantly by addition of specific proteins such as immunoglobulins and albumin (13). In addition, silica is more cytotoxic in the absence of serum, consistent with intracellular calcium changes in these two conditions. Furthermore, within this early time frame of approximately 10 min, silica was shown to stimulate intracellular kinases in the presence of serum as evidenced by the tyrosine phosphorylation results (Figure 4). Therefore, silica can cause signal transduction without an elevation of intracellular calcium. Which model (with or without serum) of macrophage stimulation is more physiological is an important consideration. While neither one is ideal, the presence of certain serum components (proteins and lipids) that may mimic those present in the lung lining fluid layer may be better than only a balanced salt solution. Furthermore, we have observed that silica-stimulated cytokine release (e.g., IL-1β and TNF-α) from human alveolar macrophages is much greater in the presence of serum.

Recent studies show that other cells such as lymphocytes can be stimulated by a tyrosine kinase-mediated pathway (24,25). Our results suggest that silica may stimulate human alveolar macrophages through such a pathway (Figure 4). We observed increased tyrosine phosphorylation following either LPS or silica stimulation of a number of proteins, most notably at 46 and 80 kDa with LPS and 46 and 50 kDa with silica. Although these proteins remain to be identified, it may be speculated that the 46-kDa protein is a MAP kinase isoform.

Our results also show that a 24-hr preincubation with silica can prime human alveolar macrophages for enhanced release of IL-1β in response to LPS (Figure 5). The effect was observed using low doses of silica and LPS and was pronounced with high doses of both silica and LPS. Consistent with the implication for the involvement of tyrosine phosphorylation in silica stimulation, MAP kinase activity was also enhanced following silica priming and correlated with the enhanced cytokine release. Therefore, silica can upregulate macrophages by some mechanism that results in increased MAP kinase activity and cytokine release to a second stimulus such as LPS. These results raise the possibility that bacterial infections could play some role in fibrogenesis.

In summary, silica increases intracellular calcium in a balanced salt solution medium. However, in the presence of 10% serum, which appears to be required for cytokine release, silica does not increase intracellular calcium levels. Silica by itself does stimulate tyrosine phosphorylation as well as prime macrophages for enhanced MAP kinase activity and cytokine release in response to a second stimulus such as LPS. Taken together, these results suggest that silica activates and modifies a tyrosine kinase pathway in human alveolar macrophages that may be important in the development of fibrosis.

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