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Modulation of Eicosanoid Production by Human Alveolar Macrophages Exposed to Silica *in Vitro*

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Repeated inhalation of silica dust can lead to inflammation and fibrosis in human lung and in experimental animal models. The alveolar macrophage is believed to play a pivotal role in this process. Numerous macrophage-derived growth factors, cytokines, and arachidonic acid metabolites have been shown to contribute to inflammation and fibrosis. The objective of this study was to determine the eicosanoid production by human alveolar macrophages in response to silica exposure *in vitro* and to assess the contribution of alveolar macrophages to silica-induced fibrosis and inflammation. Macrophages were obtained from healthy volunteers and were incubated for 3 or 24 hr in the presence of silica (100, 60, and 0 μg/mL). Supernatants were removed for eicosanoid analysis. Eicosanoids were analyzed by both high performance liquid chromatography and radioimmunoassay. The data suggest that silica causes an increased release of leukotriene B_4, leukotrienes $C_4/D_4/E_4$, and 5-hydroxyeicosatetraenoic acid (5-HETE) after 3 hr and decreases in prostaglandin E_2 and thromboxane B_2 production after 24 hr of exposure to 100 μg/mL silica. In addition, 12-HETE and 15-HETE production remained unchanged at either time point. These opposing effects seen with the metabolites of lipoxygenase and cyclooxygenase pathways could contribute to silica-induced fibrosis. The pattern of eicosanoid production after exposure to silica was different from that obtained when macrophages were stimulated with lipopolysaccharide for 3 or 24 hr, indicating that the response to the particles was not just due to general cellular activation.

Introduction

Airway inflammation and fibrosis are common features of prolonged exposure to mineral particles such as silica in humans and rodents (*1*). Silicosis is a chronic inflammatory and fibrotic lung disease caused by the inhalation of various forms of crystalline silica (silicon dioxide, SiO_2). Although much has been published about the pathogenesis of silicosis (*1*), the exact mechanism by which these particles can induce inflammatory and fibrogenic lung disease remains unclear. Increasing evidence suggests that alveolar macrophages (AM) play a key role in the onset and development of inflammatory and fibrogenic lung disease through their ability to release potent inflammatory and fibrogenic mediators, including various cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF). In addition, activated human AM have been shown to secrete growth factors capable of inducing fibroblast proliferation *in vitro* (*2*).

Another class of substances produced by AM and which is closely associated with the inflammatory response is composed of arachidonic acid (AA) metabolites or eicosanoids (*3*). Some of these metabolites possess potent proinflammatory and/or cellular regulatory activities (*3*). Prostaglandin E_2 (PGE_2), for instance, is known to inhibit fibroblast proliferation *in vitro* (*4,5*) and to augment neutrophil influx into the lung in response to complement fragments *in vivo* (*6*). Leukotriene B_4 (LTB_4) and hydroxyeicosatetraenoic acid (HETE) have also been associated with neutrophil chemotaxis at inflammatory sites including the lung (*3*). Specifically, in the context of fibrosis, LT_4 has also been shown to increase IL-1 production by human monocytes (*7*). Moreover, lipoxygenase products such as LTB_4 and leukotriene C_4 (LTC_4) were shown to promote fibroblast proliferation *in vitro* (*8*). It is possible, therefore, that eicosanoids play an important role through their direct proinflammatory and fibroblast growth regulatory properties in silica-induced lung fibrosis.

In the present study we investigated the cyclooxygenase and the lipoxygenase products (eicosanoids) produced by human AM, obtained by bronchoalveolar lavage (BAL), in response to silica exposure *in vitro* as a way to assess the contribution of AM to silica-induced pulmonary inflammation and fibrosis. The results suggest that a 3-hr exposure to silica caused a substantial release of leukotrienes B_4 (LTB_4), peptide leukotrienes C_4, D_4, and E_4

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(LTC_4, LTD_4, LTE_4), and 5-HETE. Furthermore, there was a decrease in and thromboxane production by AM after 24 hr of exposure to silica. The pattern of eicosanoid production was different from that seen after lipopolysaccharide stimulation, indicating that the response to silica is not merely due to cellular activation.

Materials and Methods

Preparation of Alveolar Macrophage Cultures

Healthy, nonsmoking male volunteers 18–35 years of age served as subjects for this study. Criteria used for selection of subjects were described earlier (*9*).

BAL was performed as previously described in detail (*9*). All aliquots were put on ice immediately after aspiration and centrifuged at 30*g* for 10 min at 4°C to separate cells from fluid. Cells from all aliquots were pooled, washed twice with RPMI 1640 (Gibco, Grand Island, NY), supplemented with 0.025% gentamicin and 2% fetal bovine serum (FBS; J.R. Scientific, Woodland, CA), and used immediately for cell culture. Cell viability exceeded 85% as ascertained by trypan blue dye exclusion. Cell differentials were performed on cytocentrifuged slides stained with modified Wright's stain. The percentage of AM ranged between 80 and 95%, 10–15% were lymphocytes, and the remainder (<3%) was made up of neutrophils, eosinophils, and epithelial cells. Cell concentration was adjusted according to the number of viable AM. Cells were plated at 65,000 cells/cm² plastic culture dishes (either 100-mm dishes or 24-well dishes) using RPMI supplemented with 2% FBS at 37°C. Nonadherent cells were removed after 1 hr. The purity of the AM in the adherent cells exceeded 97% by morphology.

Reagents

Silica particles (Minusil: Pennsylvania Glass Sand Corporation, Pittsburgh, PA) were resuspended in different concentrations in phosphate-buffered saline (PBS) containing glucose (20mM), bovine serum albumin (BSA; Sigma,St. Louis, MO) at 1 mg/mL, and $CaCl_2$ (0.1 g/L). PBS containing silica or PBS alone (control) was added to the AM cultures for 3 and 24 hr.

Before use, the silica particles were heated to 200°C for 2 hr for sterilization and inactivation of any endotoxin present. The particle size determined microscopically for 200 particles (Feret's diameters) was 2.2± 1.0 μm.

Lipopolysaccharide at 1 μg/mL (from *E. coli* serotype 026:B6; Sigma) was used to stimulate AM cultures. All complete culture media contained <0.003 ng/mL endotoxin as determined by the limulus assay (Sigma).

HPLC Analysis of Tritiated Arachidonic Acid Metabolism

For the high performance liquid chromatography (HPLC) analysis of eicosanoids, adherent AM were first incubated in 100-mm dishes with RPMI media containing 2% FBS and 5 μCi tritiated arachidonic acid (^{3}H-AA; 60-100 Ci/mmole, New England Nuclear, Boston, MA) for 18 hr. This period allows equilibration of the ^{3}H-AA within cellular lipid pools. After the ^{3}H-AA incorporation period, cells were washed twice with PBS and AM were then incubated with media alone, lipopolysaccharide (1 μg/mL), or silica particles (100 μg/mL) for 3 or 24 hr. The media was removed, the supernatants deproteinated with cold ethanol (80% v/v final concentration), the media and ethanol were then removed by rotary evaporation, and the products redissolved in 30% methanol (<80% recovery of tested eicosanoids). ^{3}H-AA metabolites were separated using a reverse-phase method using a gradient from 58 to 100% methanol over 100 min at a flow rate of 1.1 mL/min (*10,11*). Eluting radioactivity was monitored using a flow-through radioactivity detector (Radiomatic Instruments, Tampa, FL).

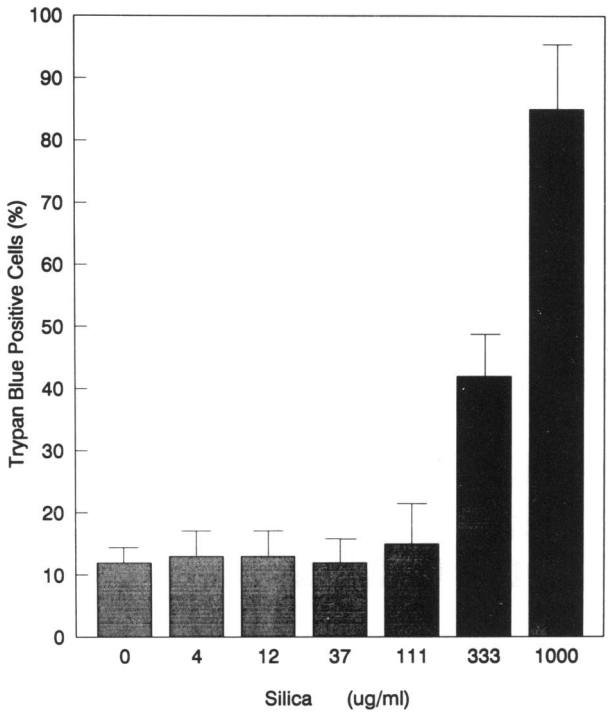

FIGURE 1. Percent trypan-blue-positive cells after exposure of alveolar macrophages (AM) to silica. AM were incubated with a range of concentrations of silica for a period of 18 hr. The viability of the cultures was then evaluated by trypan blue dye exclusion test. The bars represent changes in viablility after exposure to silica ± SD.

Measurements of Eicosanoids by Radioimmunoassay in Supernatants of Alveolar Macrophage Cultures

To determine the concentrations of individual eicosanoids, AM cultures in 24-well dishes were exposed to various stimuli for 3 or 24 hr and the supernatants from the different cultures collected and assayed by radioimmunoassay (RIA).

PGE_2 was analyzed using kits perchased from New England Nuclear using ^{125}I-PGE_2 tracer. All other RIA kits were purchased from Advanced Magnetics (Cambridge, MA) and used as tritiated tracer compound. The LTC_4 antibody had 64% and 64% cross-reactvity with LTD_4 and LTE_4, respectively. All other antibodies had <3% cross-reactivity with other eicosanoids.

Statistical Analysis

The primary hypothesis to be tested was that exposure of AM to silica *in vitro* results in a change in the production of AA

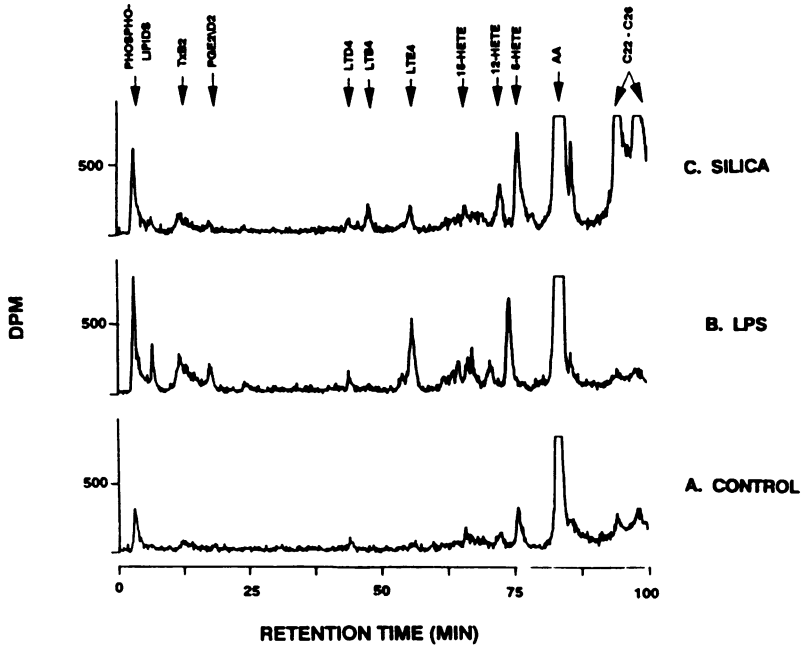

FIGURE 2. HPLC analyses of eicosanoid production by alveolar macrophages (AM) exposed to silica and lipopolysaccharide for 3 hr. Cultures of AM were labeled with ^{3}H-arachidonic acid (^{3}H-AA) for 18 hr, then incubated with media alone, lipopolysaccharide (LPS) (1 μg/mL) or silica (100 μmL) for 3 hr. ^{3}H-AA metabolites in the culture supernatants were then analyzed by HPLC (for details see Materials and Methods). TxB_2, thromboxane B_2; PGE_2, prostaglandin E_2; LT, leukotriene; HETE, hydroxyeicosatetraenoic acid.

metabolites, which could potentially be involved in the fibrogenic/inflammatory response to silica *in vivo*. The experimental design was to divide each subject's cells into two parts, one that would be incubated with medium alone, and one that would be incubated with silica. The design required that a paired *t*-test be done on the data with the measurements on control and silica exposures from each subject making up each pair. The $\log_{10}$ transformation was applied to the data before doing the *t*-test. Statistical significance was considered at $p < 0.05$. Data are reported as means ± SEM or ± SD. The data are based on six experiments ($n = 6$) performed with cells from six different individuals.

Results

Cytotoxic Effects of Silica to Alveolar Macrophages

To test the potential toxic effect of silica on AM, cultures of AM were incubated with a range of concentrations of silica (0, 4, 12, 37, 111, 333, 1000 μg/mL) for 18 hr. The viability of these cultures was then determined by the trypan blue dye exclusion test. As shown in Figure 1, silica concentrations between 4 and 111 μg/mL did not have a marked effect on the viability of the cells when compared to cells incubated without silica. In contrast, higher silica concentrations (333 and 1000 μg/mL) caused significant loss of viability, reaching 85% death at the highest concentration ($p < 0.05$). These results were confirmed by doing an independent series of experiments using the propidium iodide stain (data not shown). In subsequent experiments, therefore, only the nontoxic levels of silica were used.

HPLC Analysis of Arachidonic Acid Metabolites Produced by Silica-Exposed Alveolar Macrophages

Changes in the total spectrum of AA metabolites were assayed by HPLC. HPLC analyses of the ^{3}H-AA metabolites formed by human AM incubated with various stimuli for 3 hr are shown in Figure 2. AM incubated with media alone (Fig. 2*A*) released relatively small amounts of ^{3}H-AA metabolites into the media, mostly ^{3}H-HETEs, with free ^{3}H-AA being the major product released. In contrast, AM incubated with lipopolysaccharide at 1 μg/mL (Fig. 2*B*), a known stimulator of AA metabolism in human AM (*12*), caused the release of detectable amounts of the cyclooxygenase products ^{3}H-thromboxane B_2 and PGE_2 as the predominant product released by lipopolysaccharide-stimulated AM. AM that were incubated with silica particles at a concentration of 100 μg/mL (Fig. 2*C*) for 3 hr produced detectable amounts of ^{3}H-thromboxane B_2, ^{3}H-PGE_2/D_2, ^{3}H-A, ^{3}H-LTD_4, ^{3}H-LTE_4, ^{3}H-HETEs, and ^{3}H-AA. Additionally, two large peaks that eluted after ^{3}H-AA tentatively identified as possible elongated products of arachidonic acid (e.g., C_{22} and C_{26} fatty acids) were also detected.

Chromatograms of AM incubated for 24 hr with the same agents at the same concentrations are shown in Figure 3. Control cultures (Fig. 3*A*) produced a similar pattern of products as seen at 3 hr, with ^{3}H-AA being the predominant product produced. AM incubated with lipopolysaccharide (Fig. 3*B*) produced a large amount of ^{3}H-thromoboxane B_2 and ^{3}H-PGE_2/D_2 and the peptido-leukotrienes ^{3}H-LTD_4 and ^{3}H-LTE_4. Tritiated HETEs and ^{3}H-A were also produced in detectable amounts by the lipopolysaccharide-stimulated AM. Silica-exposed AM (Fig.

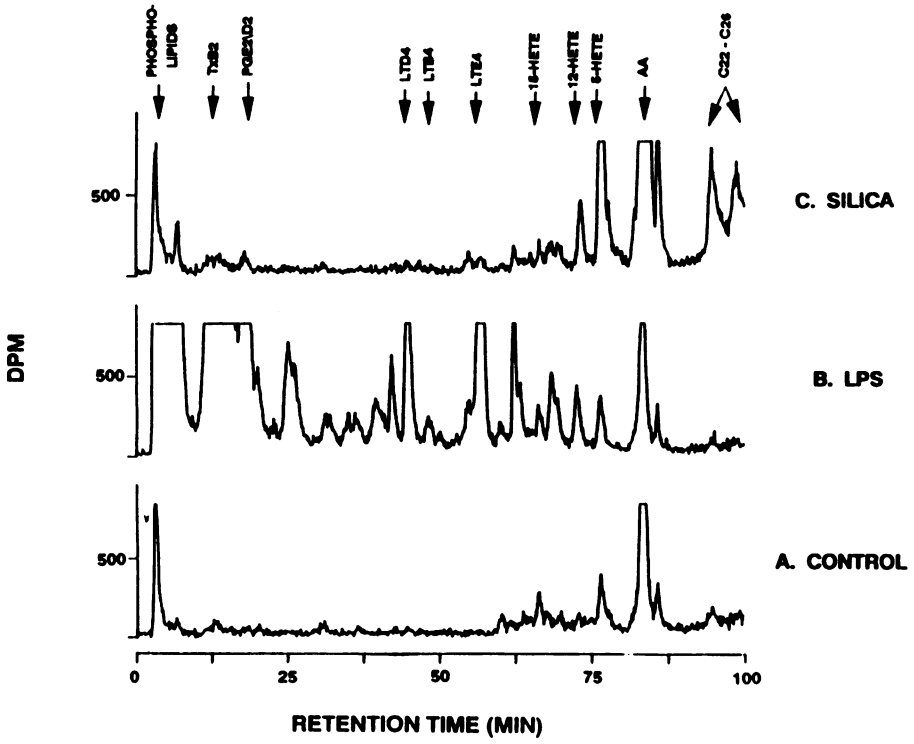

FIGURE 3. HPLC analyses of eicosanoid production by alveolar macrophages (AM) exposed to silica and lipopolysaccharide (LPS) for 24 hr. Cultures of AM were labeled with ^{3}H-arachidonic acid (^{3}H-AA) for 18 hr, then incubated with media alone, LPS (1 μg/mL), or silica (100 μg/mL) for 24 hr. ^{3}H-AA metabolites in the culture supernatants were then analyzed by HPLC (for details see "Materials and Methods"). TxB_2, thromboxane B_2; PGE_2, prostaglandin E_2; LT, leukotriene; HETE, hydroxyeicosatetraenoic acid.

3C) produced more 5-HETE after 24 hr of exposure compared to 3 hr. Free ^{3}H-15-HETE, and ^{3}H-12-HETE were present at similar concentrations at both time points. Most other tritiated products (^{3}H-thromboxane B_2, ^{3}H-PGE_2/D_2, ^{3}H-leukotrienes, ^{3}H-AA elongation products) appeared to be present in smaller amounts at 24 hr compared to the 3-hr time point.

The HPLC data suggest that stimulation of human AM by silica resulted in the release of a variety of metabolites of both the cyclooxygenase and lipoxygenase pathways with some unique peaks (elongation products) that were not present in cultures stimulated with lipopolysaccharide.

Modulation of Lipoxygenase Metabolites by Exposure of Alveolar Macrophages to Silica

To specifically quantify various AA metabolites, AM cultures were either cultured with medium alone or in the presence of silica for 3 or 24 hr and metabolites measured by RIA. The selection of eicosanoids to be assayed by RIA was based on the HPLC findings (Figs. 2 and 3).

LTB_4, mainly associated with chemotactic activity for neutrophils, was elevated in the 3-hr silica-exposed cultures in a dose-dependent manner, reaching a statistically significant level ($p < 0.05$) at 60 and 100 μg/mL (Fig. 4). In contrast, the 24-hr cultures were not statistically different from control (data not shown). LTC_4, similar to LTB_4, was significantly elevated in response to a 3-hr exposure to silica at 100 and 60 μg/mL (Fig. 5). Another metabolite of the 5-lipoxygenase pathway, 5-HETE, was significantly elevated compared to controls at 3 and 24 hr

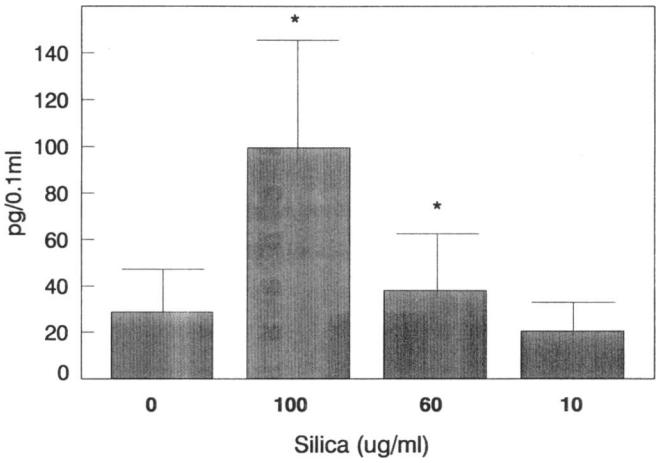

FIGURE 4. The effect of silica exposure on leukotriene B_4 (LTB_4) release from cultures of alveolar macrophages (AM). Cultures of AM were stimulated with various concentrations of silica for 3 hr. Levels of LTB_4 were determined in culture supernatants by radioimmunoassay. Bars represent levels of LTB_4 detected in the supernatants ± SEM. (*) p-Value of 0.05 or less.

after stimulation with 100 and 60 μg/mL silica but not at 10 μ/mL. Interestingly, though, the control levels of 5-HETE after 24 hr dropped < 10-fold compared to the 3-hr time point (Fig. 6).

Two metabolites of the 12- or 15-lipoxygenase pathway, namely, 12-HETE and 15-HETE, have also been detected by RIA with similar patterns to those found by HPLC (Figs. 2 and 3).

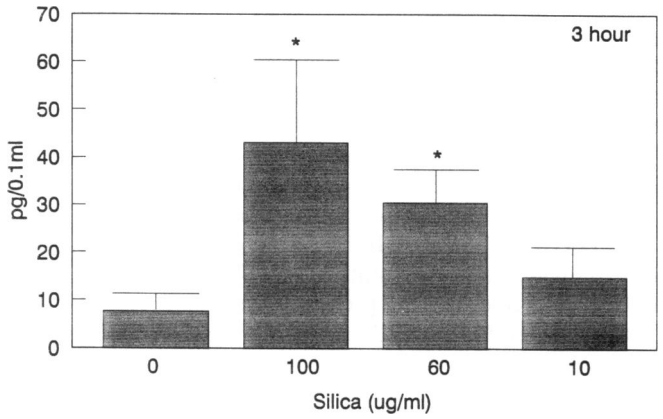

FIGURE 5. The effect of silica exposure on leukotrienes $LTC_4/D_4/E_4$ release from alveolar macrophages (AM). Cultures of AM were stimulated with various concentrations of silica for 3 hr. Levels of $LTC_4/D_4/E_4$ were determined in culture supernatants by radioimmunoassay. Bars represent levels of $LTC_4/D_4/E_4$ detected in the supernatants ± SEM. (*) p-Value of 0.05 or less.

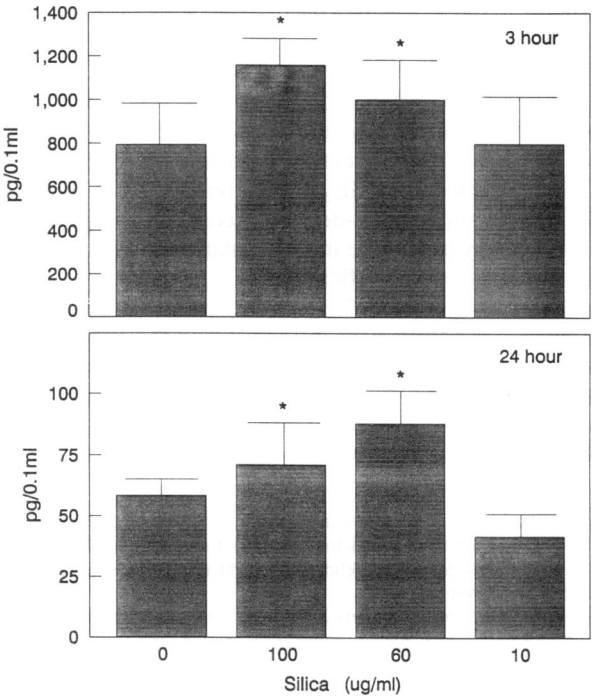

FIGURE 6. The effect of silica exposure on 5-hydroxyeicosatetraenoic acid (5-HETE) release from alveolar macrophages (AM). Cultures of AM were stimulated with various concentrations of silica for 3 and 24 hr. Levels of 5-HETE were determined in culture supernatants by radioimmunoassay. Bars represent levels of 5-HETE detected in the supernatants ± SEM. (*) p-Value of 0.05 or less.

Silica did not seem to significantly affect their levels at either time point (data not shown).

Modulation of Cyclooxygenase Metabolites by Exposure of Alveolar Macrophages to Silica

Two metabolites of the cyclooxygenase pathway detected by HPLC analysis were also measured by RIA. Thromboxane B_2

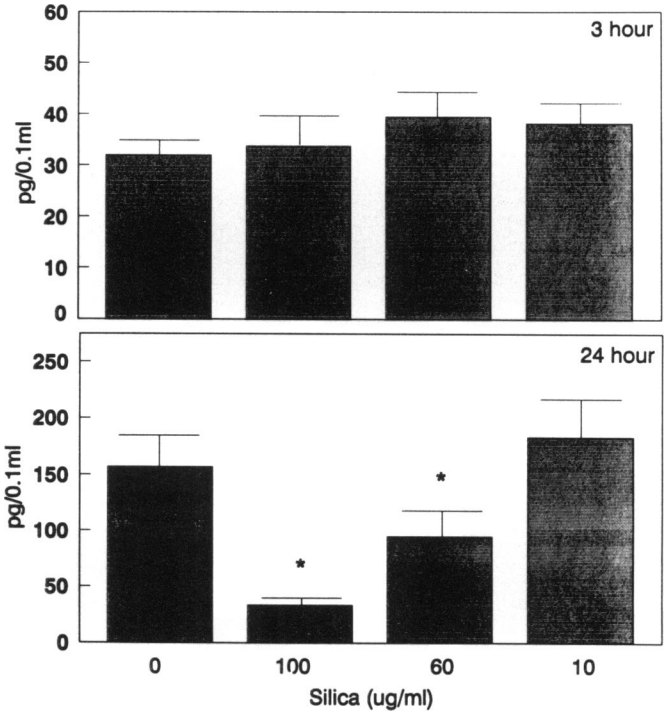

FIGURE 7. The effect of silica exposure on thromboxane B_2 release from alveolar macrophages (AM). Cultures of AM were stimulated with various concentrations of silica for 3 and 24 hr. Levels of thromboxane B_4 were determined in culture supernatants by radioimmunoassay. Bars represent levels of thromboxane B_2 detected in the culture supernatants ± SEM. (*) p-Value of 0.05 or less.

levels were not changed with any other silica concentrations used after 3 hr but did show a significant decrease below the control level after 24 hr of exposure to silica at 100 and 60 μg/mL (Fig. 7). PGE_2 levels were unchanged after a 3-hr exposure to silica but were significantly depressed compared to control at the 100 and 60 μg/mL concentrations after 24-hr exposure (Fig. 8).

Discussion

Fibrosis is a prominent feature of advanced silicosis. The macrophage has been implicated as the cause of the fibrosis that accompanies silicosis. Normal animal macrophages exposed to silica *in vitro* have a variable effect on fibroblast function, apparently quite dependent on the culture conditions established (*6,13*). Many chemical messengers are probably involved in the complex cellular events that lead to fibrosis, and factors such as interferons, interleukins, fibronectin, and macrophage-derived growth factors are thought to be important (*14*). AA metabolites, PGE_2 and LTB_4, are potent proinflammatory and /or cellular regulatory modulators (*15*). The AM is a rich source of many of these eicosanoids, yet the role of these compounds in mediating pulmonary inflammation and fibrosis has only recently begun to be investigated.

In our initial studies we established the range of silica concentrations that would not cause death of the cells. The data showed that concentrations exceeding 100 μg/mL of silica were clearly cytotoxic to human AM *in vitro*. The cytotoxic effect of silica has been thought to be a major component in the development of

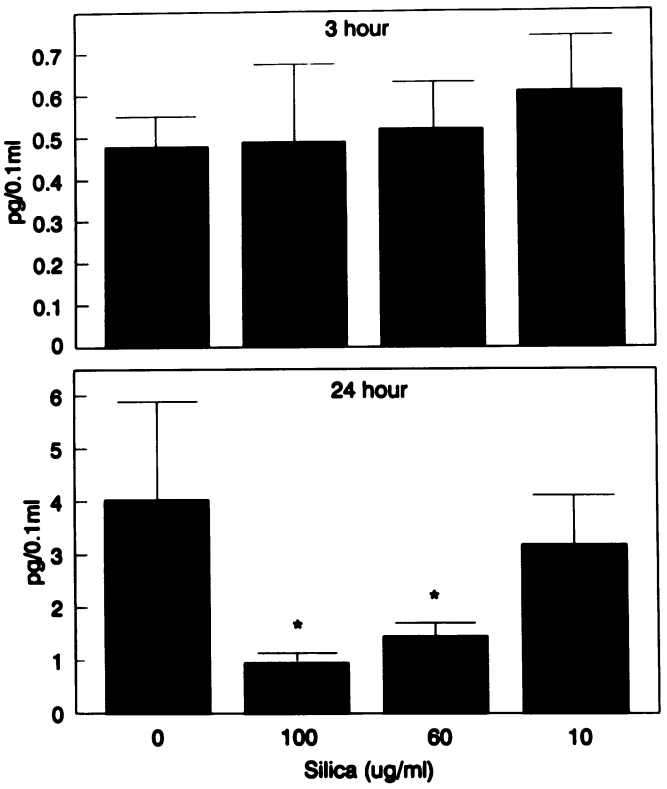

FIGURE 8. The effect of silica exposure on prostaglandin E_2 (PGE_2) release from alveolar macrophages (AM). Cultures of AM were stimulated with various concentrations of silica for 3 and 24 hr. Levels of PGE_2 were determined in culture supernatants by radioimmunoassay. Bars represent levels of PGE_2 in the culture supernatants ± SEM. (*) p-Value of 0.05 or less.

fibrosis (*16*). It seems, however, that the response of AM to the ingestion of silica in a nontoxic range is dose dependent. Therefore, the results reported here represent biological responses to silica at nontoxic concentrations of silica.

The HPLC analysis of supernatants collected from human AM stimulated with silica (at 100 μg/mL) revealed a broad spectrum of eicosanoids produced after 3 hr of exposure, with lesser amounts of the cyclooxygenase metabolites and an increased amount of some lipoxygenase products seen at the later time point (24 hr). The levels of the AA metabolites were increased in the silica-exposed cultures compared to the controls and were distinct from those obtained by stimulation with lipopolysaccharide (1 μg/mL), which is known to be a potent stimulator of eicosanoids in macrophages (*17*). The unique products released by exposure of AM to silica, thought to be elongated products of AA (C_{22}–C_{26}), have not been thoroughly characterized as far as their possible biological functions.

In agreement with the HPLC analysis, RIA demonstrated increased levels ot LTB_4 after 3 hr of silica exposure. This finding also correlates agreement with a previous study using human AM (*17*) and recent studies using rat (*18*) and bovine (*14*) AM. LTB_4 has previously been shown to be chemotactic for leukocytes (*19,20*). In addition, LTB_4 has been shown to enhance the release of TNF, which can promote fibroblast growth (*18,21*), and modulate other cytokines such as IL-1, which may also be involved in the fibrotic process (*8,22*). The other lipoxygenase products, $LTC_4/D_4/E_4$ and 5-HETE, were also elevated in response to the silica exposure at 60 and 100 μg/mL, but not at the lowest concentration of 10μg/mL tested. HETEs in general have been shown to have chemotactic activity for leukocytes (*23*), and LTC_4 has been suggested to be involved in the regulation of fibroblast growth (*8*). Two other lipoxygenase products, 12- and 15-HETE, were not affected by the exposure of the AM to silica.

The increased production of 5-lipoxygenase products, i.e., $LTC_4/D_4/E_4$ LTB_4, and 5-HETE, in response to silica exposure *in vitro* suggests a specific stimulation of this enzyme's activity, as 12- and 15-lipoxygenase products were not increased.

Interestingly, PGE_2 and thromboxane B_2, two metabolites of the cyclooxygenase pathway, were downregulated by silica after 24 hr and were not changed at the earlier time point. The PGE_2 production in response to silica described here agrees well with the findings of Brown et al. (*2*) using cytotoxic levels of silica (1000 μg/mL) for a duration of 24 hr. PGE_2 has been shown to turn off the secretion of various AM-derived cytokines (e.g., TNF and IL-l) involved in fibrosis (*17,22*). The potential relevance of thromboxane B_2 to fibroblast proliferation is currently unknown. The decrease in two cyclooxygenase products in response to silica suggests a decrease in cyclooxygenase activity in silica-exposed cells.

Taken together, the data suggest that those AA products that enhance fibroblast growth and inflammation (i.e., lipoxygenase metabolites) are increased in response to stimulation of AM with silica, whereas those products associated with suppression of fibroblast growth (e.g., PGE_2) are decreased. Thus, the ability of silica to modulate AM-derived eicosanoids suggests that eicosanoids may play a role in inflammation and fibrosis associated with inhalation of silica.

Further research needs to be performed to further elucidate the mechanisms responsible for the changes in the enzymatic activities of the eicosanoid cascade. Work is currently in progress to determine possible changes in the levels of various cytokines at the protein and mRNA levels that may contribute to the overall mechanism responsible for silica-induced fibrosis.

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The research described in this article has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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