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Acute Pulmonary Responses in Vivo to Silica Complexed with H^+, Zn^{2+}, or Fe^{3+}

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This investigation is to determine whether the surface complexation of iron influence acute pulmonary responses induced by silica. For this study, three varieties of cation complexed silica were used: silica-H^+, -Zn^{2+}, and -Fe^{3+}, since the first two are not active in the transport of electrons and generate little free radicals relative to the dust with the surface iron. Rats (270 to 280 g) were intratracheally (IT) instilled with saline, silica-H^+, -Zn^{2+}, or -Fe^{3+}(5 mg in 0.5 ml saline). After 4 h, cell number, type, and differentiation were analysed in the bronchoalveolar lavage cells, and the levels of lactate dehydrogenase (LDH) and total protein were determined in the lavage fluid. In addition, bronchoalveolar lavage cells were cultured, and nitric oxide production was measured using nitrate assay. Inducible nitric oxide synthase (iNOS) mRNA in the bronchoalveolar lavage cells was also determined by northern blot analysis. Differential counts of the lavage cells showed that red blood cells were increased by 9-, 8-, and 13-fold and total leukocytes (lymphocytes plus polymorphonuclear neutrophils) by 48-, 36-, and 33-fold, following IT silica- H^+, -Zn^{2+}, and -Fe^{3+}, respectively compared with the saline group. Meanwhile, there were no significant differences in red blood cells and total leukocytes among any of the cation complexed silica groups. The levels of LDH and total protein in the lavage fluid were significantly increased by 3- to 4-fold. However, compared among these silica groups, Fe^{3+} complexation did not significantly change the LDH activity and total protein. NO production in cultured bronchoalveolar lavage cells was elevated by 2-fold, following IT any of the silica treatments compared with the saline group. Furthermore, the steady-state levels of iNOS mRNA in the lavage cells were greatly increased. There were any differences in iNOS mRNA expression among the silica-treated groups as with NO production. These findings suggest that surface complexed iron may not influence the acute pulmonary responses resulted from 4h exposure to silica.

Key Words: Pulmonary responses, Silica, iNOS mRNA, NO, Fenton reaction

INTRODUCTION

Occupational exposure to silica can result in the development of pulmonary fibrosis. Cellular injury and tissue damage are believed to be important steps in the development of silicosis (Davis, 1986). Damage to the lung parenchyma may result from the direct toxicity of silica or indirectly from the generation of reactive oxygen species (ROS) by pulmonary phagocytes (Lapp & Castranova, 1993).

In an aqueous environment, silica (SiO_2) is covered with surface hydroxyl groups, and silica surface has some concentration of silanol groups (-Si-OH) to coordinate ferric iron (Laszlo, 1987; Ghio et al, 1992). The ferric ion (Fe^{3+}) complexed by the surface of this dust allows silica to function as Fenton catalysts, resulting in the production of · OH by an electron exchange via the Fenton reaction (Cohen G, 1985):

$$Fe^{3+} + e^- \rightleftharpoons Fe^{2+} \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightleftharpoons Fe^{3+} \cdot OH + OH^- \quad (2)$$

The ability of silica to generate oxidants via the

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Fenton reaction has been established in vitro, indicating a role for iron in ROS generation by silica (Kennedy et al, 1989). A role for iron in vivo generation of ROS by silica has also been demonstrated (Kamp, 1992). Therefore, complexation of inorganic and body sources of iron on silica surfaces may result in oxidant generation (Ghio, 1990).

Recent evidence suggests that alveolar macrophage- and neutrophil-derived nitrogen free radicals may play important roles in mediative cytotoxicity through damping DNA and inhibiting the mitochondrial respiratory cycle (Stuehr & Nathan, 1989; Wink et al, 1993). In the previous study, it was found that inducible nitric oxide synthase (iNOS) mRNA expression and nitric oxide production by alveolar macrophages and recruited neutrophils where increased in acute inflammatory lung injury following exposure to silica. Nitric oxide (NO) may also combine with superoxide anion (oxidant generated via the Fenton reaction) to produce an even more cytotoxic molecule, peroxinitrite ($ONOO^-$) (Koppenol et al, 1992). Peroxinitrite is a long-lived, reactive oxidant that contributes to inflammatory tissue damage. In addition, cytotoxic effects of NO can be explained by its interaction with iron. It has been demonstrated that the loss of activity of the critical Krebs-cycle enzyme aconitase in activated macrophages is a result of the formation of iron-nitrosyl complexes (Drapier & Hibbs, 1988; Lancaster & Hibbs, 1990).

The present study, examines whether surface complexed with Fe^{3+} influences silica-induced pulmonary responses, which 1) elicit acute lung inflammation and injury in rats, and 2) stimulate NO production and iNOS mRNA induction by bronchoalveolar lavage cells obtained from silica-exposed rats. For this study, three varieties if silica were used: complexed with H^+, Zn^{2+} and Fe^{3+}, since H^+- and Zn^{2+}-complexed silica are not active in the transport of electrons and generate little free radicals relative to that dust with the surface iron (Ghio et al, 1992).

METHODS

Preparation of surface complexed silica

Three varieties of cation-complexed silica dusts (silica complexed with H^+ (silica-H^+), Zn^{2+} (silica-Zn^{2+}), and Fe^{3+} (silica-Fe^{3+})) were prepared by Dr. A.J. Ghio (Duke University, U.S.A.) and delivered for this study. In the present study, the relationship between the ability in the transport of electrons of these surface cations and silica-induced pulmonary responses was examined. Briefly, silica (2 mg/ml) was stirred in solutions of either 1 mM HCl, $ZnCl_2$, or $FeCl_3$ for 15 min to provide each cation-loaded silica, respectively. They were then centrifuged at 1,200 g for 10 min and washed 10 times in water. Silica was then dried at 37°C for 4 days. In addition, Ghio et al (1992) determined the ability of silica to further complex Fe^{3+} from solutions of 1.0 mM $FeCl_3$ where silica was found to have significantly elevated cheatable iron concentrations of 61.3 ± 1.5 μM/g.

Experimental design of four groups

The experimental design consisted of four groups of rats (specific pathogen-free male Sprague-Dawley rats weighing 260~290 g): (1) a saline-treated group that received an intratracheal (IT) instillation of 0.5 ml of LPS-free saline (0.9% NaCl); (2) a silica-H^+-; (3) a silica-Zn^{2+}-; and (4) a silica-Fe^{3+}- treated group which received IT instillation of 5 mg silica either complexed either with H^+, Zn^{2+}, or Fe^{3+} in 0.5 ml saline, respectively.

Intratracheal instillation

Rats were anesthetized with intraperitoneal injection of 0.5 ml of a 1% solution of sodium methohexital. After that, they were intratracheally instilled with saline, silica-H^+, silica-Zn^{2+}, or silica-Fe^{3+} using 20-gauge, 4-inch ball-tip animal feeding needle (Brain et al, 1976).

Isolation of bronchoalveolar lavage cells and cell counts

4 h after treatment, the rats were anesthetized with 0.2 g/kg body weight of sodium pentobarbital. The trachea was then cannulated and the lungs were lavaged 10 times with 8 ml aliquots of Ca^{++}, Mg^{++}-free Hanks balanced salt solution (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, and 5.5 mM dextrose; pH=7.4). The bronchoalveolar lavagate was centrifuged (500×g for 5 min), and the bronchoalveolar lavage cells were washed and resuspended in Hepes-buffered solution (145 mM NaCl, 5 mM KCl, 10 mM Hepes, 5.5 mM dextrose, and 1.0 mM $CaCl_2$; pH= 7.4). Cell counts and differentials were determined

using an electronic coulter counter with a cell sizing analyzer (Coulter Model ZBI with a channelizer 256; Coulter Electronics, FL, USA), as described by Lane & Mehta (1990). Red blood cells (RBC), lymphocytes, polymorphonuclear neutrophils, and alveolar macrophages were distinguished by their characteristic cell volumes (Castranova et al, 1990).

Lactate dehydrogenase (LDH) activity and total protein measurement

The LDH activity and total protein were measured in the first aliquot of the acellular bronchoalveolar lavage fluid. The activity of LDH, a cytosolic enzyme used as a marker for cytotoxicity, was measured by the method of Cabaud & Wroblewsli (1958). Using bovine serum albumin as a standard, the measurement of total protein was performed according to the method of Hartreea (1972) to assess the permeability of the bronchoalveolar-capillary barrier.

Nitrate assay in cultured bronchoalveolar lavage cells

Bronchoalveolar lavage cells were suspended in MEM (medium essential medium) with 10% fetal bovine serum, 2 mM glutamine, 100 u/ml penicillin, and 100 μg/ml streptomycin at a final concentration of 1×10^6 alveolar macrophages/ml. From cell suspension, 1 ml aliquots were added to 24 well plates (Castar, MA, USA) and incubated for 2 h at 37°C in a humidified atmosphere of 5% CO_2. The nonadherent cells were then removed with two 1 ml washes of the fresh MEM. After incubating for 24 h, the cell cultures were centrifuged at 500 g for 15 min and the supernates frozen at −70°C until they were assayed.

To measure total nitrate (nitrite plus nitrate), nitrate in the samples was first reduced to nitrite with nitrate reductase, and the nitrite reaction was then determined with Greiss reagent. Nitrite was assayed after adding 100 μl Greiss reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 50 μl samples of cell culture. Optical density at 550 nm (OD_{550}) was measured using a microplate reader. Nitrite concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in cell culture medium and presented as nmole/mg protein.

Total RNA isolation and northern blot analysis

The bronchoalveolar lavage cells from individual rats were centrifuged ($500\times g$ for 10 min). Total cellular RNA was extracted by a modification of the acidic guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi, 1987). The RNA pellet was vacuum-dried for 1.5 h and resuspended in 0.5% sodium dodecyl sulfate (SDS). The RNA was quantified in a spectrophotometer at 260 nm. Total cellular RNA (20 μg) was diluted in loading buffer (N-morpholinopropane-sulfonic acid [MOPS], 40% formamide, and 15% formaldehyde). The RNA was electrophoresed on a 5.6% formaldehyde denaturing 1% agarose gel. The RNA was vacuum-transferred onto a Nytran membrane. The membrane was washed with $2\times$SSC, dried at 65°C, and then UV cross-linked. The membrane was prehybridized at 68°C for 30 min with 5 ml Quickhyb® hybridization buffer plus 200 μl of 10 mg/ml herring sperm DNA boiled for 10 min. Subsequently, the ^{32}P-α-dCTP labeled murine macrophage iNOS ssDNA probe (5×10^7 cpm) was added, and the hybridization was performed for 1 h at 68°C. The membrane was washed at a low stringency 3 times with 50 ml of $2\times$SSC, 0.1% SDS at room temperature; washed again at high stringency for 30 min with 50 ml of $0.2\times$SSC, 0.1% SDS at 60°C; and finally apposed to Kodak X-ray film at −70°C.

Statistics data were expressed as means ± SD

Mean values of cellular differential, protein contents, LDH activity, and nitrate production were compared by one-way ANOVA. Significance was set at $p\leq0.05$. For each dependent variable, two separate analyses were conducted: one which included saline vs Si-H^+, Si-Zn^{2+}, or Si-Fe^{3+}; and the other which included Si-H^+, Si-Zn^{2+}, and Si-Fe^{3+}.

RESULTS

To evaluate and compare acute inflammatory lung injuries of rats exposed to silica complexed with H^+, Zn^{2+}, or Fe^{3+}, cell differential in bronchoalveolar lavage cells, and LDH activity and protein contents in bronchoalveolar lavage fluids were analysed.

Differential analysis of bronchoalveolar lavage cells showed that the number of alveolar macrophages

slightly decreased after 4 h exposure to silica-H^+ or silica-Fe^{3+} below the value saline-treated group, whereas in silica-Zn^{2+} group, the number of alveolar macrophages was significantly decreased ($p \leq 0.05$) (Fig. 1). However, there were no significant differences in the number of alveolar macrophages among any of the silica- H^+, -Fe^{3+}, and -Zn^{2+} ($p \leq 0.05$) groups.

Compared with the saline group, the number of red blood cells was significantly increased by 9-, 8-, and 13-fold, and total leukocytes also increased (lymphocytes plus polymorphonuclear neutrophils) by 48-, 36-, and 33- fold, following IT silica-H^+, silica-Zn^2, and silica-Fe^{3+}, respectively ($p \leq 0.05$) (Fig. 1). However, there were no significant differences in red blood cells and total leukocytes among the rats treated with these cation complexed silica ($p \leq 0.05$).

As shown in Fig. 2 and 3, the LDH activity and protein contents in the lavage fluid were significantly increased by about 3-4 fold in silica complexed with H+, Zn^{2+}, or Fe^{3+} compared with the saline group ($p \leq 0.05$), whereas there were no significant changes among these silica-treated groups ($p < 0.05$).

Increasing evidences suggest that a large part of silica-induced alveolar damage is due to the production of alveolar macrophage- and neutrophil-derived cytotoxic species such as NO. Cytotoxic effects of NO can be explained by its interaction with iron. Therefore, the relationship between surface iron complexation and the ability of silica to induce NO production as well as iNOS mRNA expression by alveolar macrophages and neutrophils was examined.

Nitrate, which is an oxidation end product of nitric

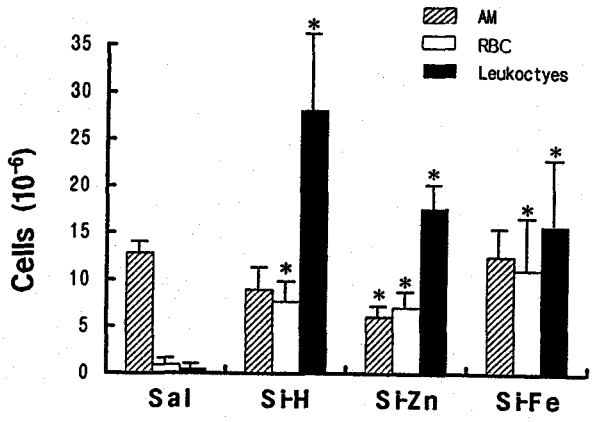

Fig. 1. Cell counts and differentials in bronchoalveolar lavage cells. 4 h after it instillation with saline, silica-H^+, -Zn^{2+}, or -Fe^{3+} (5 mg in 0.5 ml saline), bronchoalveolar lavage cells were counted and differentiated using an electronic coulter counter with a cell sizing analyzer. Alveolar macrophages (AM), red blood cells (RBC), and leukocytes (lymphocytes plus polymorphonuclear neutrophils) were counted and distinguished by their characteristic cell volumes. Values represent means ± SEM (n=4). *Significant difference compared with control (it saline instilled), $p \leq 0.05$.

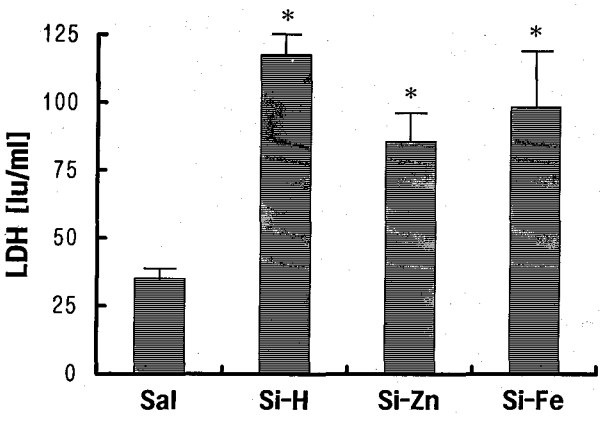

Fig. 2. The activity of LDH present in bronchoalveolar lavage fluid. LDH was measured in bronchoalveolar lavage fluid 4 h after it instillation of saline, silica-H^+, -Zn^{2+}, or -Fe^{3+} (5 mg in 0.5 ml saline). Values represent means ± SEM of results from 4 experiments. *Significant difference compared with control(it saline instilled), $p \leq 0.05$.

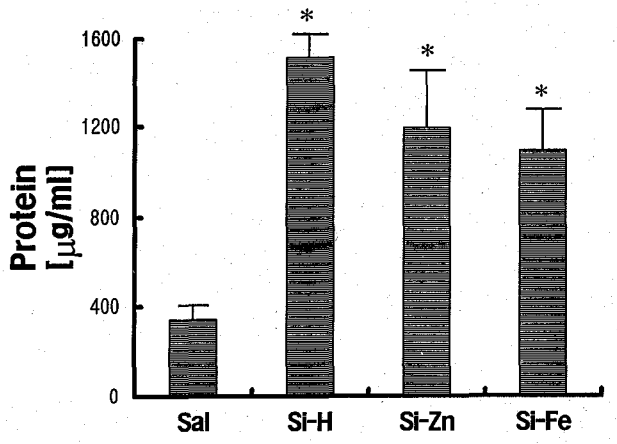

Fig. 3. Total protein content in bronchoalveolar lavage fluid. Rats were it instilled with saline, silica-H^+, -Zn^{2+}, or -Fe^{3+} (5 mg in 0.5 ml saline). After 4 h, protein content in the bronchoalveolar lavage fluid was measured spectrometrically at 560 nm. Values represent means ± SEM of results from 4 experiments. *Significant differences compared with control (it saline instilled), $P \leq 0.05$.

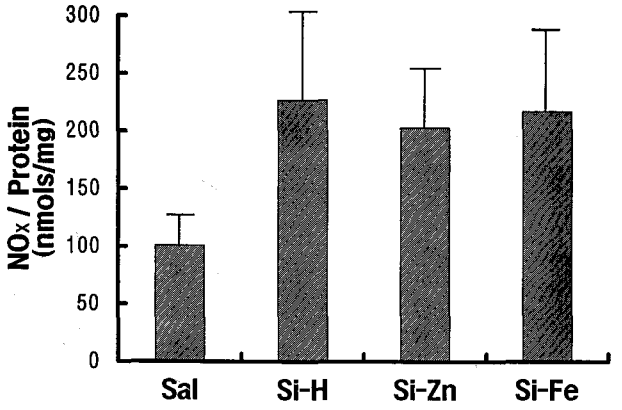

Fig. 4. Nitric oixde (NO) production in the cultured bronchoalveolar lavage cells. 4 h after it instillation with saline, silica-H^+, -Zn^{2+}, or -Fe^{3+} (5 mg in 0.5 ml saline), bronchoalveolar lavage cells were cultured for 24 h and no production was measured using nitrate assay. Values represent means ± SEM of results from 4 experiments. *Significant difference compared with control (it saline instilled), $p \leq 0.05$.

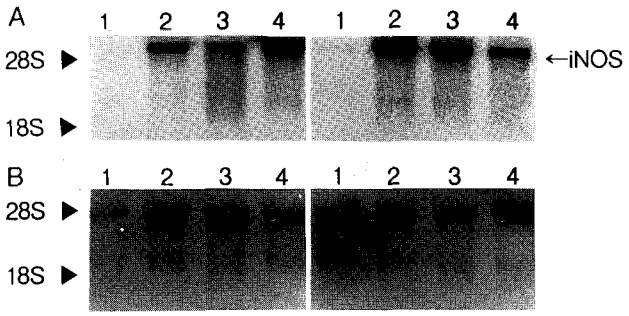

Fig. 5. Northern analysis in bronchoalveolar lavage cells showing autoradiographs of blots probed with murine macrophage iNOS ssDNA probe (A) and methylene blue staining of the rRNA (B). Treatment groups include saline (1); silica-H^+ (2); silica-Zn^{2+} (3); silica-Fe^{3+} (4).

oxide, was measured in bronchoalveolar lavage cells cultured for 24 h after exposure to silica-H^+, Zn^{2+}, or Fe^{3+}. Treatment of any one of the complexed silica increased nitrate by 2-fold above the level of saline control (≤ 0.05) (Fig. 4). Fig. 5 shows a Northern blot demonstrating an increased iNOS-specific mRNA expression after treatment with silica-H^+, Zn^{2+}, or Fe^{3+}, which indicates up-regulation of iNOS mRNA steady state levels in bronchoalveolar lavage cells. However, Fe^{3+} complexation of silica did not enhance silica ability to stimulate NO production as well as iNOS mRNA expression by bronchoalveolar lavage cells.

DISCUSSION

The results show that intratracheal instillation of each of silica complexed with H^+, Zn^{2+}, or Fe^{3+} caused increase in the number of red blood cells and total leukocytes (lymphocytes plus polymorphonuclear neutrophils). In contrast, there was a decrease in the number of alveolar macrophages compared with the saline group. Since activated macrophages after exposure to silica may adhere more tightly to the air ways and be more difficult to remove by lavage (Willoughby et al, 1986). It was also found that the LDH activity and total protein contents were significantly increased in bronchoalveolar lavage fluid obtained from rats exposed to any of surface complexed silica, compared with the saline-treated group. However, compared between rats exposed to the surface complexed silica, Fe^{3+} complexation onto the silica surface did not significantly change the differential cell counts in bronchoalveolar lavage cells and the LDH activity and total protein contents in the lavage fluid. Theses data indicate that the ability of silica itself to induce damage to the lung parenchyma and the alveolar-capillary carrier was not influenced by surface complexation of Fe^{3+}. In contast to these findings, Ghio et al (1992) have demonstrated silica complexed with Fe^{3+} greatly increased both the cellular influx, including the number of neutrophils, and protein contents in the large fluid obtained from the rats after 96h relative to wetted silica. This disagreement may be due to the time exposed to silica, i.e., 4 h in the present study vs 96 h in the study by Ghio et al (1992). Recently, Ghio et al (1998) have characterized changes in the concentrations of ferritin and lactoferrin in the lower respiratory tract after exposure of rats to an emission source air pollution particle. The highest concentration of these iron-binding proteins occurred at approximately 24 h after exposure to the air pollution particle, and then the concentration started to decrease. By 96 h after instillation, the concentration was not elevated relative to those animals exposed to saline. Therefore, 4 h of exposure as performed in the present study rather than 96 h may be more appropriate to determine whether surface complexed metal is responsible for silica-induced pulmonary inflammation because when the exposure time delayed over 96 h, more intricated factors reflecting the host environment such as kine-

tics of the concentration of iron-binding proteins may be involved in the experimental system.

NO production in these 24 h cultured cells was also significantly increased compared with the saline-treated rats. Increases in iNOS mRNA steady-state levels in the lavage cells including mainly alveolar macrophages and polymorphonuclear neutrophils were also increased following exposure to silica- with H^+, Zn^{2+}, or Fe^{3+}. As with the data from NO production, the optical density analysis of iNOS bands in the lavage cells shows no significant differences in iNOS steady-state levels among rats treated with silica-H^+, Zn^{2+}, and Fe^{3+}(data not shown), indicating that Fe^{3+} complexation of silica did not enhance silica ability to stimulate iNOS mRNA expression as well as NO production by bronchoalveolar lavage cells. These results are supported by the findings of Kamp et al (1992), where iron was not required to generate the reactive radical, suggesting silica also appears to catalyze electron transfer reactions which do not require iron.

It is concluded that surface complexed Fe^{3+} does not influence acute lung inflammation and NO production as well as iNOS expression by alveolar macrophages and neutrophils resulted from 4 h exposure to silica.

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